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Review

Use of HIV as a gene transfer vector

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Despite the extensive research efforts over the past 25 years that have focused on HIV, there is still no cure for AIDS. However, tremendous progress in the understanding of the structure and biology of the HIV virus led to the development of safe and potent HIV-based transgene delivery vectors. These genetic vehicles are referred to as lentiviral vectors. They appear to be better suited for particular applications, such as transgene delivery into stem cells, compared to other viral- and non-viral vectors. This is because Lentivirus-based vectors can efficiently infect non-dividing and slowly dividing cells. In the present review article, the current state of understanding of HIV-1 is discussed and the main characteristics that had an impact on vector design are outlined. A historical view on the vector concept is presented to facilitate discussion of recent results in vector engineering in a broader context. Subsequently, a state of the art overview concerning vector construction and vector production is given. This review also touches upon the subject of lentiviral vector safety and related topics that can be helpful in addressing this issue are discussed. Finally, examples of Lentivirus-based gene delivery systems and their applications are presented, with emphasis on animal transgenesis and human gene therapy.

Keywords: Lentivirus, lentiviral vectors, AIDS, HIV-1 life cycle; viral-host protein interactions, gene therapy, animal transgenesis, cell engineering

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Abbreviations: AIDS, acquired immunodeficiency syndrome; APC, antigen presenting cell; bGHpA, bovine growth hormone polyadenylation sequence; BL2, Biosafety Level 2 (containment); CA, capsid protein; CaP, DNA/calcium phosphate coprecipitation; CFU/ml, colony forming units per ml; cHS4, chicken hypersensitive site 4 sequence (β-globin insulator); CMV, cytomegalovirus; CNS, central nervous system; cPPT, central polypurine tract; CTS, central termination sequence; DC, dendritic cell; ΔU3, self-inactivating deletion in U3 region of 3' LTR; DIS, viral RNA dimerization initiation site (dimerization signal); Dox, doxycycline; DSE, distal sequence element; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; Env, envelope glycoprotein; FACS, fluorescence activated cell sorting; gRNA, viral genomic RNA; HBS, HEPES-buffered saline; HEK 293T, human embryonic kidney 293T cells; HIV, human immunodeficiency virus; HOS, human osteosarcoma cells; HSPC, hematopoietic stem/progenitor cell; IDLV, integration-deficient lentiviral vector; IN, viral integrase; iPS, induced pluripotent stem cell; IRES, internal ribosome entry sequence; KRAB, Krüppel-associated box (silencer domain); LEDGF/p75, lens epithelium-derived growth factor; LTR, viral long terminal repeat; MA, matrix protein; MLV, murine leukemia virus; MOI, multiplicity of infection; NC, nucleocapsid protein; NLS, nuclear localization sequence; NPC, nuclear pore complex; PBS, primer binding site; PEI, poly(ethylene imine); PIC, viral preintegration complex; polyA (pA), polyadenylation signal; PPT, polypurine tract; PR, viral protease; Pr55, Gag precursor protein; Pr160, Gag-pol precursor protein; PSE, proximal sequence element; ψ, viral RNA packaging signal; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; R, LTR repeat element; RCL, replication competent lentivirus; RNAP II, RNA polymerase II; RNAP III, RNA polymerase III; RRE, Rev response element; RT, reverse transcriptase; RTC, viral reverse transcription complex; rtTA, reverse Tet-controlled transactivator; SA, splice acceptor site; SD, splice donor site; shRNA, small hairpin RNA; SIN, self-inactivating (vector); siRNA, small interfering RNA; SIV, simian immunodeficiency virus; SU, gp120 surface subunit; TAR, transactivation response element; Tet, tetracycline; tetR, Tet repressor; TM, gp41 transmembrane subunit; TRE, Tet-responsive element; tTA, Tet-controlled transactivator; tTS, Tet-controlled transcriptional repressor; TU/ml, transducing units per ml; U3, LTR 3′ unique element; U5, LTR 5′ unique element; UTR, untranslated region; VSV-G, vesicular stomatitis virus G glycoprotein; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

INTRODUCTION

The 2008 Nobel Prize in Physiology or Medicine was shared by three clinician scientists who had worked with two important viral pathogens, human papilloma virus (HPV) and human immunodeficiency virus (HIV). Harald zur Hausen was awarded the prize "for his discovery of HPVs causing cervical cancer" and Françoise Barré-Sinoussi together with Luc Montagnier — "for their discovery of HIV". The discovery of HPV (Dürst *et al.*, 1983) eventually led to the development of safe and effective vaccines. The first such vaccine, Silgard/Gardasil (Merck/Sanofi Pasteur MSD), was licensed in EU countries by the European Medicines Agency (EMEA) in 2006 and the second one, Cervarix (Glaxo Smith Kline, GSK), in 2007 (source: EMEA; see List of websites [1]). In contrast, over twenty five years after the link between HIV and the acquired immunodeficiency syndrome (AIDS) was demonstrated (Barré-Sinoussi *et al.*, 1983), no efficacious vaccine for HIV is in sight (for comments see: Veljkovic *et al.*, 2008; Amanna & Slifka, 2009).

Although prophylactic vaccines are yet to be formulated, the intensive and detailed studies on HIV biology helped to design effective antiviral drugs, such as CCR5-receptor antagonists (Lieberman-Blum *et al.*, 2008) or integrase inhibitors (Cocohoba & Dong, 2008), and led to the establishment of clinical protocols, introduced in 1996, referred to as "highly active antiretroviral therapy (HAART)" (for current reviews see: Flessa & Marschall, 2009; Bowman *et al.*, 2009; Richman *et al.*, 2009; for complete list of drugs used in the treatment of HIV infection, approved by the U.S. Food and Drug Administration (FDA), see List of websites [2]).

HIV and other lentiviruses (a genus of the *Retroviridae* family; for taxonomy details see: *The Universal Virus Database* (ICTVdB), authorized by the International Committee on Taxonomy of Viruses (ICTV); List of websites [3] and [4]), due to their unique features, have emerged as a powerful tool for transgene delivery into cells and for generation of transgenic animals. Moreover, HIV-based vectors have reached the clinic as promising vehicles for anti-viral gene therapy or vaccine applications.

Viral vectors have gained their popularity in basic research and gene therapy applications because of their high rates of gene transfer that are far superior to those achieved with non-viral methods. Numerous types of virus-derived gene transfer systems are available these days. These genetic vehicles are based either on DNA viruses or on RNA viruses. The corresponding vectors either integrate into the host genome or express their genetic information episomally.

Retroviral vectors, due to their ability to integrate into the host DNA, are widely used in both cell biology and biomedicine. Retrovirus-based systems (for a review see: Buchschacher, 2001), unlike episomal viruses (herpes simplex virus — HSV, adenovirus), offer stable, long-term transgene expression. Simple retroviruses (so-called gamma- or oncoretroviruses) play an important role in the modern biomedicine. More than 21% of gene therapy protocols approved worldwide until March 2009 (1537 trials) employed retroviral vectors and, among all viral and non-viral methods, only adenovirus-based vectors are currently more popular (about 24%) (data provided by *J Gene Med*; see List of websites [5]).

A serious drawback of using onco-retroviruses in clinical trials is their natural disposition to integrate near promoters and regulatory regions (Wu *et al.*, 2003; De Palma *et al.*, 2005; Tsukahara *et al.*, 2006; Deichmann *et al.*, 2007; reviewed in Daniel & Smith, 2008) and to induce insertional tumors due to the presence of potent transcriptional enhancers in viral long terminal repeats (LTRs). In fact, such an adverse outcome has been reported earlier in two X-linked severe combined immunodeficiency (X-SCID1) patients treated with cytokine-activated hematopoietic stem/progenitor cells (HSPCs) transduced with murine leukemia virus — MLV (trial initiated in 1999) (Hacein-Bey-Abina *et al.*, 2003). More recently, further two cases with T cell leukemia developed after this gene therapy trial have been described. A detailed study has revealed that in all four patients blast cells harbor activating vector insertions near the LIM domain-only 2 (*LMO2*) and *BMI1* proto-oncogenes (Hacein-Bey-Abina *et al.*, 2008). Not surprisingly, in a more recent attempt to correct the same immune dysfunction (trial initiated in 2001), one of ten patients successfully treated has developed leukemia due to *LMO2* overexpression as a result of an adjacent vector integration (Board of the European Society of Gene and Cell Therapy, 2008). Also here the MLV vector was used, but in a different transduction protocol. In a Swiss-German gene therapy trial started in 2006 — correction of chronic granulomatous disease (X-CGD) — expansion of clones by insertional activation of growth-promoting genes by spleen focus-forming virus (SFFV) was observed (Ott *et al.*, 2006). Disease complications have caused death of one of three successfully treated patients (European Society of Gene Therapy (ESGT), 2006).

Another limitation to the application of gamma-retroviral vectors is that they can infect only dividing cells. This is because viral preintegration complex (PIC), a large nucleoprotein complex responsible for viral cDNA integration, cannot enter the cell nucleus and requires disassembly of the nu-

clear envelope during mitosis (Roe *et al.,* 1993; Lewis & Emerman, 1994).

These drawbacks can be avoided by using lentiviral vectors. Lentiviruses have evolved different strategies to interact with the host cell chromatin and do not integrate preferentially into close proximity of transcription start sites but rather favor introns in chromosomal regions rich in expressed genes (Schroder *et al.,* 2002; Mitchell *et al.,* 2004; De Palma *et al.,* 2005; Laufs *et al.,* 2006; Yang *et al.,* 2008a; Felice *et al.,* 2009; Wang *et al.,* 2009a). This discrepancy in the integration site selection between simple retroviruses and their more complex relatives could be explained by the different interactions of host cellular factors with viral integrase (IN) and Gag-derived proteins (Lewinski *et al.,* 2006). One of such host proteins responsible for proviral integration, lens epithelium-derived growth factor (LEDGF/p75), interacting with HIV-1 IN, has been identified (Cherepanov *et al.,* 2003). LEDGF accounts for the characteristic propensity of Lentivirus to integrate within active transcription units and is required for efficient viral replication (for review see: Engelman & Cherepanov, 2008; Ciuffi, 2008).

Another advantage of Lentivirus-based vectors, important for research and gene therapy applications, is their ability to infect both dividing and nondividing cells. This feature, unique among retroviruses, substantially increases the range of cells available for gene transfer. Lentiviral vectors transduce quiescent cells, including primary hepatocytes (Pfeifer *et al.,* 2001; Zamule *et al.,* 2008; Dagher *et al.,* 2009), progenitor and stem cells (Reiser *et al.,* 1996; Miyoshi *et al.,* 1999; Ricks *et al.,* 2008; Santoni de Sio & Naldini, 2009), nonproliferating monocytes and macrophages (Weinberg *et al.,* 1991; Naldini *et al.,* 1996a**;** Zufferey *et al.,* 1997; Su *et al.,* 2008; Veron *et al.,* 2009) as well as postmitotic neurons (Naldini *et al.,* 1996a; 1996b; Blömer *et al.,* 1997; Zufferey *et al.,* 1997**;** Wong *et al.,* 2004; Hioki *et al.,* 2009; Federici *et al.,* 2009).

The first recombinant retroviral vector systems, with a replication-defective virus, were developed in the early 1980s (Mann *et al.,* 1983; Watanabe & Temin, 1983). Seven years later, and seven years after the discovery of HIV, an early system for recombinant HIV-1-based vector production was described (Page *et al.,* 1990; Landau *et al.,* 1991). In this system replication-defective HIV-1 vector with the sequences encoding the viral envelope (Env) glycoprotein deleted was cotransfected with expression vectors encoding heterologous Env proteins (ecotropic and amphotropic MLV Env, or human T cell leukemia virus type I — HTLV-I Env) to form HIV-1 pseudotypes. Although during the last two decades HIV-1-based vectors became accepted as the most promising gene delivery tool among lentiviruses, also other members of the genus serve as platforms for recombinant vector generation. To date, constructs based on genomes of the following lentiviruses have been developed: human immunodeficiency virus 2 (HIV-2) (Poeschla *et al.,* 1998a), various simian immunodeficiency viruses (SIVs) (Nègre *et al.,* 2000; Nakajima *et al.,* 2000), feline immunodeficiency virus (FIV) (Poeschla *et al.,* 1998b), bovine immunodeficiency virus (BIV) (Berkowitz *et al.,* 2001a), caprine arthritis-encephalitis virus (CAEV) (Mselli-Lakhal *et al.,* 1998), equine infectious anemia virus (EIAV) (Olsen, 1998), Jembrana disease virus (JDV) (Metharom *et al.,* 2000) and Maedi-Visna virus (MVV) (Berkowitz *et al.,* 2001b).

Lentiviral vectors, due to their unique properties, including selection of "safe" integration sites in the host genome, efficient long-term gene delivery to both dividing and nondividing cells, relatively large cargo capacity (7–8 kb without affecting vector titer) and target specificity achieved by pseudotyping are promising agents in research and medicine. However, their origin from mammalian pathogens, especially those developed from HIV viruses, raises the question of safety and this matter needs careful consideration.

This article presents a brief overview on the Lentivirus–host interactions and describes problems associated with the design of safe and efficacious lentiviral vectors, methods of production and purification of high titer virus preparations, strategies of delivery of genetic information to the diverse eukaryotic cells using pseudotyped lentiviruses as well as applications of recombinant lentiviral vectors in animal transgenesis and human gene therapy.

HIV-1 — BASIC VIROLOGY

AIDS

On June 5, 1981, the *Morbidity and Mortality Weekly Report* (MMWR) published information from the Centers for Disease Control and Prevention (CDC) about five homosexual men in Los Angeles treated for *Pneumocystis carinii* pneumonia (PCP) (CDC, 1981). This was the first published notice of what, a year later, became known as AIDS. Since the beginning of the epidemic, 25 million people have died of HIV-related causes. Thus, HIV is one of the leading causes of death worldwide and it leads to higher mortality than any other infectious disease.

From a clinical point of view, HIV is the most relevant Lentivirus. According to UNAIDS' *2008 Report on the global AIDS epidemic*, there were an estimated 33 million (30–36 million) people living with HIV globally in 2007. The annual number of new

HIV infections in 2007 was 2.7 million. Overall, 2 million people died due to AIDS in 2007, compared with an estimated 1.7 million in 2001 (data available from UNAIDS; see List of websites [6]).

We do not intend to review the medical conditions that are connected with AIDS in detail. Progression of HIV-related disease, epidemiology and treatment, have been recently discussed by others (Klimas *et al.*, 2008; Wainberg & Jeang, 2008; Weiss, 2008; Ho & Bieniasz, 2008). Briefly, an initial, acute phase of HIV infection is followed by gradual immune exhaustion. HIV infects CD4+ lymphocytes as well as a variety of other cells, including monocytes and thymocytes, *via* interactions with cell surface molecules, such as CD4 receptor and chemokine coreceptors (CXCR4, CCR5). Since CD4+ cells (T helper cells) play a key role in the immune system by activating other cells such as the cytotoxic T cells and the B cells, their depletion from mucosal-associated lymphoid tissue (MALT) and blood circulation initially leads to minor infections, including cold sores (herpes simplex), condyloma (warts) and fungal infections, thrush and vaginal candidiasis, followed by serious opportunistic infections and cancers typical of AIDS.

The virulence and pathogenesis of HIV is reviewed thoroughly by Forsman and Weiss (2008). While the answer as to why HIV is a pathogen is obvious: the virus is a parasite that destroys the host immune system, there is not an easy answer as to why HIV eventually kills us. It is not clear how the high virulence of the pathogen might help in increasing its transmissibility. It is a paradox that HIV-1 is highly pathogenic in humans, whereas its precursor in chimpanzees, simian immunodeficiency virus (SIVcpz), is not. Infection of chimpanzees with HIV-1 also does not cause AIDS, so it is mostly the host rather than the virus that determines whether the disease occurs. The second human AIDS virus, HIV-2, derived from non-pathogenic SIVsm of the sooty mangabey, is less virulent in humans than HIV-1, but those individuals who develop AIDS do so with symptoms similar to HIV-1 infection. A better understanding of HIV's biology and mechanisms that govern non-pathogenic infection with SIV in chimpanzees and mangabeys might eventually lead to new approaches to treating AIDS (reviewed by: Liovat *et al.*, 2009; Sodora *et al.*, 2009). It is particularly important due to the fact that transmissions of new HIV strains to humans from novel hosts (gorillas) are still being reported (Plantier *et al.*, 2009).

VIRUS

Suggestions that the virus responsible for AIDS belongs to the *Lentivirinae* (Lentivirus) genus of family *Retroviridae* appeared soon after its discovery, when some structural and antigenic similarities to equine infectious anemia virus (EIAV) (Montagnier *et al.*, 1984) and sequence homology to Maedi-Visna virus (MVV) (Sonigo *et al.*, 1985; Gonda *et al.*, 1985) were reported. Lentiviruses means "slow" viruses, so named because the course of infection is characterized by a long interval between the initial infection and the onset of serious symptoms.

Lentiviruses have more complex genomes than simple retroviruses and employ sophisticated mechanisms that control all steps of infection. Some of these mechanisms rely on higher-order structures of the viral genome that, until very recently, were only poorly understood. The architecture and secondary structure of an entire HIV-1 genome, at single nucleotide resolution, has been recently published by Weeks and co-workers (Watts *et al.*, 2009). For the first time, we can appreciate the structural complexity of the viral genomic RNA.

Lentiviral genome consists of two linear positive-sense single-stranded RNA molecules. The dimeric nature of the HIV-1 RNA genome is largely responsible for the high genetic variability of the viruses due to possible recombinations during reverse transcription (Rhodes *et al.*, 2003). After its conversion into cDNA, in the act of viral invasion of the host cell, it becomes integrated into chromosomal DNA as a provirus (the size of the HIV-1 provirus is about 9.7 kb; details can be found in *HIV Sequence Compendium 2009* published on-line; see List of websites [7]) and is transmitted through daughter cell generations upon cell division. As an independent transcription unit, it has its own regulatory elements and is transcribed by the cell's transcription system. Figure 1A shows the structural features of HIV-1 provirus.

The protein-encoding regions are flanked by 5′ and 3′ LTRs, which consist of 3′ unique elements (U3), repeat elements (R) and 5′ unique elements (U5), and harbor some of the *cis*-acting elements. These *cis* elements contain signals important for provirus integration into the host genome (*att* repeats, which are located at the 5′ and 3′ ends of provirus DNA), enhancer/promoter sequences, transactivation response element (TAR) and polyadenylation signal (polyA). Besides the two LTRs there are other *cis*-acting sequences including the primer binding site (PBS); viral RNA packaging/dimerization signals (ψ and DIS); central polypurine tract (cPPT) and the central termination sequence (CTS), leading to the formation, during reverse transcription, of a three-stranded DNA structure called the central DNA Flap. In addition, there is the Rev response element (RRE) that is essential for post-transcriptional transport of unspliced and incompletely spliced viral mRNAs from the nucleus to cytoplasm and the purine-rich region (polypurine tract; PPT), which pro-

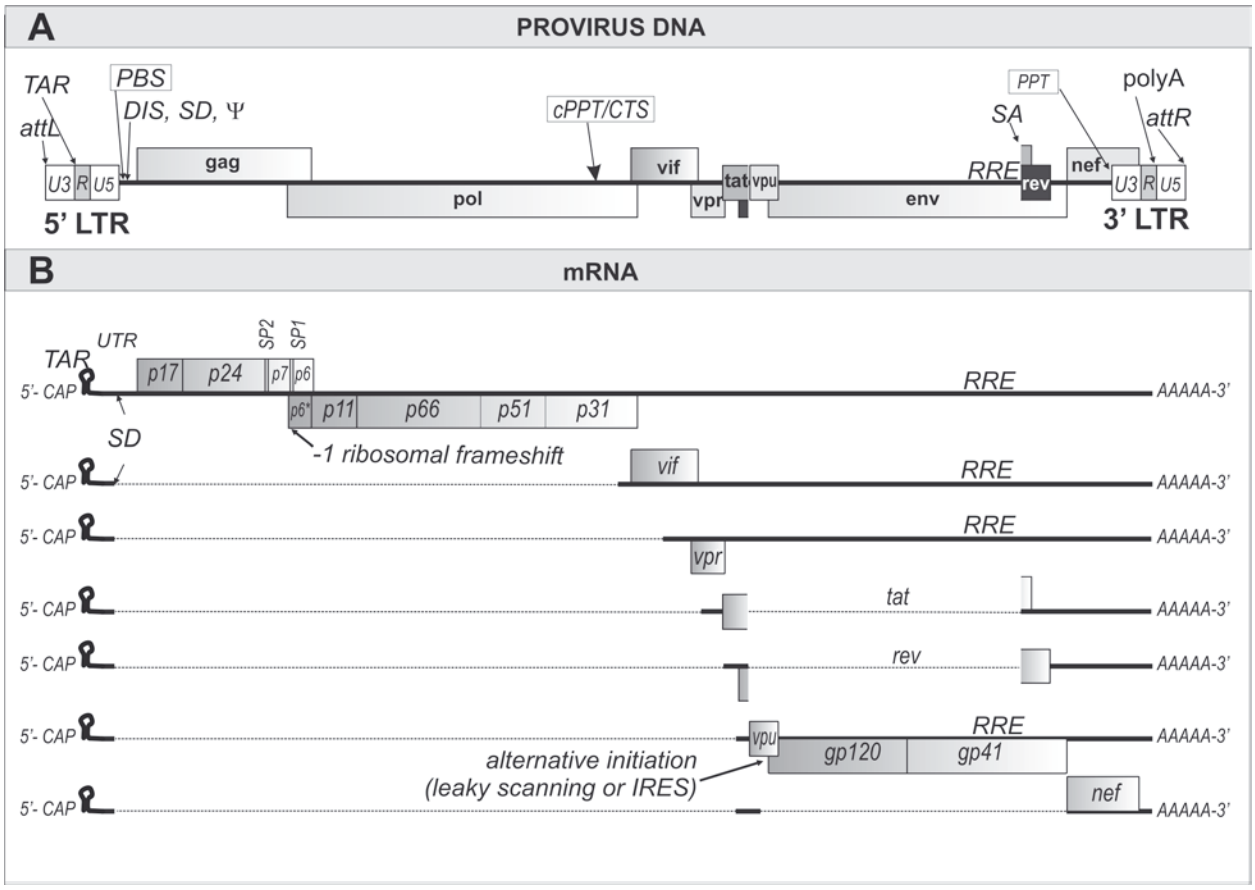

Figure 1. Schematic representation of HIV-1 genome features.
A. Elements present in the provirus. Rectangles (except LTRs) represent open reading frames positioned according to frame number (1, 2, 3) above, on, and below the line, respectively. The *cis* elements crucial for replication (PBS, cPPT, CTS, PPT) and integration (*att*L, *att*R) of the virus, transcription (U3, TAR, polyA), splicing (SA, SD) and export of unspliced transcripts (RRE), for dimerization (DIS) and packaging (ψ) of genomic RNA are indicated. **B**. Viral mRNA products resulting from provirus transcription and RNA splicing. Three classes of viral mRNAs are present in infected cells: approx. 9.2 kb, approx. 4 kb and approx. 1.8 kb. Rectangles represent expected final protein products that result from transcription, RNA splicing and protease cleavage of protein precursors. Ribosomal frameshift and alternative translation initiation sites are indicated.
Abbreviations: LTR, long terminal repeat; *att*L, *att*R, left and right attachment sites; U3, 3′ unique element; R, repeat element; U5, 5′ unique element; TAR, transactivation response element; PBS, primer binding site; DIS, dimerization signal; SD, splice donor site; SA, splice acceptor site; ψ, packaging signal; cPPT, central polypurine tract; CTS, central termination sequence; RRE, Rev response element; PPT, 3' polypurine tract; polyA, polyadenylation signal; UTR, untranslated region; cap, terminal 7-methylguanosine; IRES, internal ribosome entry sequence; AAAAAA, polyA tail.

vides a second RNA primer for the initiation of plus strand DNA synthesis by virus-specific reverse transcriptase (reviewed by: Srinivasakumar, 2001; Spirin *et al.*, 2008; for more information about structural features of HIV-1 genome see also: *HIV Sequence Compendium 2009* — List of websites [7]).

The *trans* elements of the HIV-1 provirus include nine open reading frames (ORFs). The *gag-pol*, *gag* and *env* ORFs are encoding structural proteins and enzymes that are typical of all retroviruses. Additional ORFs code for essential regulatory proteins (*tat* and *rev* genes) and accessory proteins (genes: *vif*, *vpu*, *vpr*, and *nef*). Due to the compact size of the viral genome various strategies are used for expression of its genetic information (Fig. 1). There are nineteen protein products encoded by the provirus (summarized in Table 1). In almost all retroviruses, 5′ LTR drives transcription of an initial genome-length RNA that also acts as an mRNA for translation of the viral Gag and Pol proteins. The Gag precursor protein (Pr55) is proteolytically cleaved by viral protease to yield the matrix (MA; p17), capsid (CA; p24), nucleocapsid (NC; p7), p6 and two spacer polypeptides: SP1 and SP2 (reviewed by Bukrinskaya, 2007). The Gag-pol precursor protein (Pr160), resulting from ribosomal slippage on a highly structured SLIP element (–1 frameshift) into the overlapping *pol* ORF, is cleaved by the same protease resulting in the p6* transframe (TF) polypeptide and three viral enzymes: protease (PR; p11), reverse transcriptase with ribonuclease H (RNase H) activity (RT; heterodimer p66/p51 and RNase H; p15), and integrase (IN; p31).

Table 1. HIV-1 proteins and their functions

Category	Gene/Origin	Protein	Protein function	References
structural	*gag*/ viral protease-mediated cleavage of Gag polyprotein (Pr55) during virion maturation	matrix (MA; p17)	nuclear import of viral PIC, export of Gag–RNA complex to cytosol, membrane binding, virion (coats inner leaflet of viral envelope) and PIC formation	Gallay *et al.*, 1995; 1996; reviewed by: Scarlata & Carter, 2003; Bukrinskaya, 2007; Ganser-Pornillos *et al.*, 2008
		capsid (CA; p24)	membrane binding and capsid formation (core shell)	reviewed by: Scarlata & Carter, 2003; Bukrinskaya, 2007; Ganser-Pornillos *et al.*, 2008
		nucleocapsid (NC; p7)	membrane binding, virion and PIC formation (coats genomic RNA), RNA binding and cofactor of RT, genomic RNA selection, packaging and dimerization during virion assembly	reviewed by: Scarlata & Carter, 2003; Bukrinskaya, 2007; Ganser-Pornillos *et al.*, 2008; Mougel *et al.*, 2009
		SP1	regulation of cleavage rate, membrane binding and virion formation	reviewed by: Bukrinskaya, 2007; Ganser-Pornillos *et al.*, 2008
		SP2	regulation of cleavage rate, membrane binding, virus budding and virion formation	reviewed by: Bukrinskaya, 2007; Ganser-Pornillos *et al.*, 2008
		p6	Vpr recruitment to virion, PTAP motif engaged in interactions with host proteins, membrane binding and virion formation	Paxton *et al.*, 1993; Salgado *et al.*, 2009; reviewed by: Scarlata & Carter, 2003; Bukrinskaya, 2007; Ganser-Pornillos *et al.*, 2008
	gag-pol/ cleavage of Gag-pol polyprotein (Pr160) by PR during virion maturation	p6* (transframe polypeptide; TF)	stabilization of Gag–Gag-pol interactions and activation of PR-mediated cleavage	Chiu *et al.*, 2006
enzymes		reverse transcriptase and RNase H (RT: heterodimer p66/p51 and p15)	synthesis of viral cDNA (reverse transcriptase enzymatic activity with ribonuclease H activity located at C-terminus of p66 subunit)	reviewed by: Basu *et al.*, 2008; Mougel *et al.*, 2009
		protease (PR; p11)	cleavage of p160, virion maturation	reviewed by: Boden & Markowitz, 1998; Louis *et al.*, 2007
		integrase (IN; p31)	nuclear import of viral PIC, viral cDNA integration into host genome	Gallay *et al.*, 1997; Woodward *et al.*, 2009; reviewed by Poeschla, 2008
envelope glycoproteins	*env*/ cleavage of Env (gp160) protein by cellular proteases	gp120 (SU)	interaction with host cell CD4 receptor and coreceptors	reviewed by Melikyan, 2008
		gp41 (TM)	anchors gp120/gp41 complex in virus membrane, membrane fusion with host cell	reviewed by: Weissenhorn *et al.*, 2007; Melikyan, 2008
regulatory proteins	*tat*	transactivator (Tat; p16/p14)	initiation of transcription from viral LTR, transcriptional elongation, chromatin remodeling, regulation of apoptosis, modulator of host immune response	reviewed by: Seelamgari *et al.*, 2004; Barboric & Peterlin, 2005; Bolinger & Boris-Lawrie, 2009; Cheng *et al.*, 2009
	rev	regulator of expression of viral proteins (Rev; p19)	control of proviral DNA integration, stimulation of nuclear export of unspliced HIV RNAs, stimulation of translation of HIV RNAs	Levin *et al.*, 2009; reviewed by: Cullen, 2003; Seelamgari *et al.*, 2004; Suhasini & Reddy, 2009; Groom *et al.*, 2009
accessory proteins	*nef*	negative factor (Nef; p27)	down-regulation of CD4 receptor, down-regulation of MHC-I and MHC-II, signal transduction interference, enhancing infectivity (actin cytoskeleton rearrangement)	Stumptner-Cuvelette *et al.*, 2001; reviewed by: Seelamgari *et al.*, 2004; Anderson & Hope, 2005; Foster & Garcia, 2008
	vif	viral infectivity factor (Vif; p23)	inactivation of host immune response *via* binding apolipoprotein B mRNA editing enzyme (APOBEC3 proteins) and interferon regulatory factor, IRF3	reviewed by: Seelamgari *et al.*, 2004; Barraud *et al.*, 2008; Okumura *et al.*, 2008
	vpr	viral protein R (Vpr; p14)	nuclear import of viral PIC, control of fidelity of reverse-transcription, cell cycle arrest (block of G2/M transition), induction of apoptosis, transactivation of HIV LTR and host cell genes, modulation of host immune response, inhibition of cellular and viral pre-mRNA splicing	reviewed by: Seelamgari *et al.*, 2004; Le Rouzic & Benichou, 2005; Andersen *et al.*, 2008; Majumder *et al.*, 2009; Zhang & Aida, 2009; Okumura *et al.*, 2008
	vpu	viral protein U (Vpu; p16)	degradation of CD4 receptor, release of viral particles, regulation of apoptosis	Neil *et al.*, 2008; reviewed by: Seelamgari *et al.*, 2004; Nomaguchi *et al.*, 2008

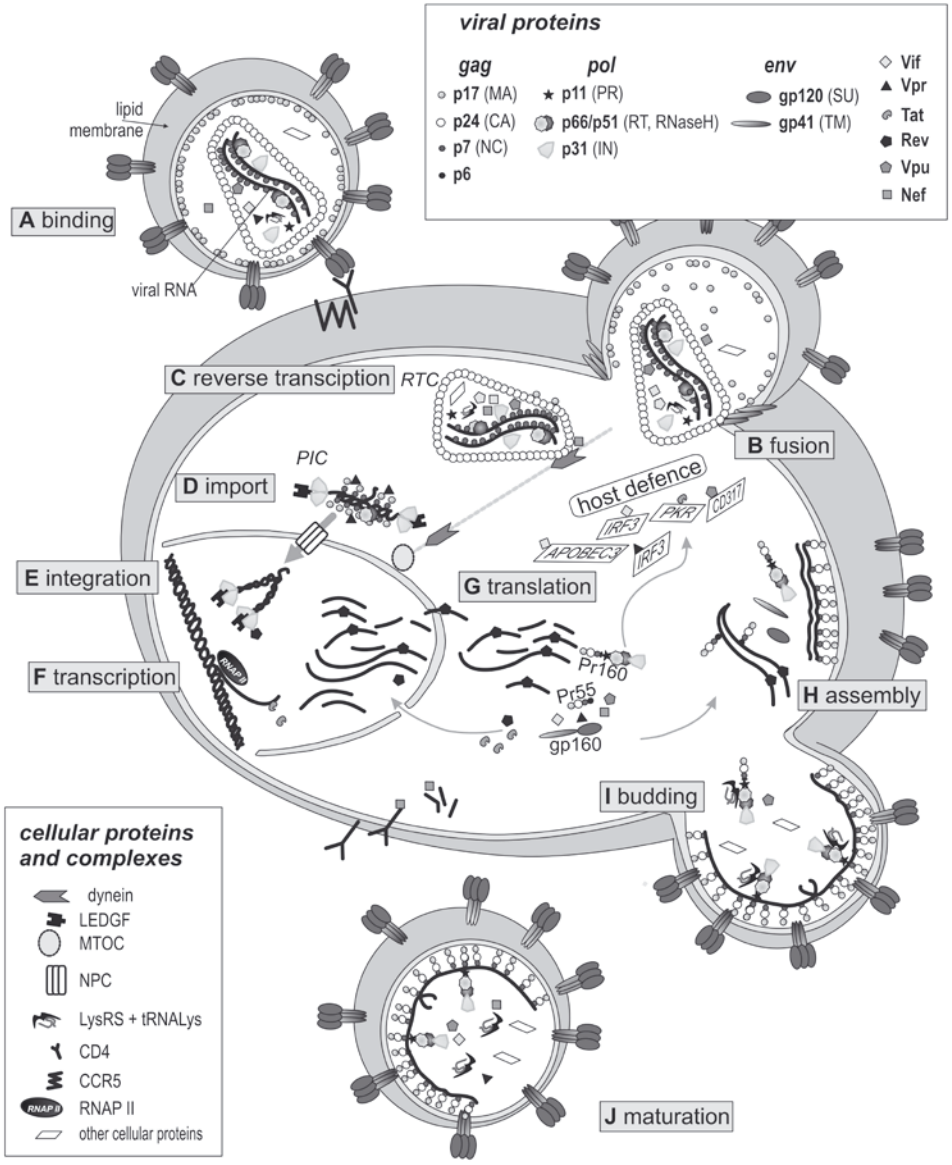

Figure 2. Scheme of HIV-1 life cycle.
Virus replication starts when the viral envelope glycoprotein gp120 binds to the CD4 receptor and a secondary receptor on the host cell surface (**A**). After binding to CD4, conformational changes in non-covalently associated gp41 subunit releases free energy sufficient to promote fusion of the virus particle with cell membrane. The viral core is then spilled into the cytoplasm (**B**). Core movement is facilitated by Nef protein which induces actin rearrangements. Reverse transcription of viral RNA occurs within CA capsid. Cellular protein dynein transports the reverse transcription complex (RTC) towards the nucleus along microtubules anchored to microtubule organizing center (MTOC) (**C**). When the reverse transcription is completed, provirus DNA and several viral proteins such as RT, IN, NC, Vpr, and MA, together with cellular proteins, form preintegration complex (PIC), which now can be actively transported into the nucleus *via* nuclear pore complex (NPC) (**D**). Proviral DNA is integrated into the host genome (**E**) by integrase, which cooperates with cellular protein LEDGF. Transcription of provirus is conducted by cellular RNAP II and enhanced by viral protein Tat. Unspliced and partially spliced transcripts are stabilized by viral protein Rev (**F**), which enables their export to the cytoplasm and subsequent translation (**G**). Some of the synthesized viral proteins participate in combat against host defense. Full-length RNA molecules, Gag, Gag-pol and Env proteins, start to assemble new virus particles *via* Gag dimerization and multimerization, binding of Gag complexes to genomic viral RNA (gRNA) and association with cellular membrane. Env precursor gp160 is cleaved by cellular proteases to give gp120 and gp41 subunits that are directed to the cell membrane by Gag (**H**). Initially, virus particles are released (**I**) from infected cell as an immature form (**J**). Digestion of polyproteins by viral protease leads to virus maturation (**A**).
Abbreviations: MA/p17, matrix protein; CA/p24, capsid protein; NC/p7, nucleocapsid protein; p6, viral p6 protein; PR/p11, protease; RT/p66/p51, reverse transcriptase with RNase H activity; IN/p31, integrase; SU/gp120, surface glycoprotein; TM/gp41, transmembrane glycoprotein; Tat, Rev, regulatory proteins; Vif, Vpr, Vpu, Nef, accessory proteins; Pr160, Gag-pol precursor polyprotein; Pr55, Gag precursor polyprotein; gp160, envelope (Env) glycoprotein; RNAP II, RNA polymerase II.

In more complex retroviruses including lentiviruses, this initial transcript is also processed into fully spliced transcripts encoding the Tat and Rev regulatory proteins as well as the auxiliary protein Nef. Alternatively, in HIV-1 this transcript can be processed into partially spliced mRNAs coding for the three other accessory proteins: Vif, Vpu and Vpr (Vpx, a Vpr homolog present in HIV-2 and some SIVs is absent in HIV-1). These partially processed transcripts also contain the *env* ORF for envelope glycoprotein (Env, gp160) translated thanks to the leaky scanning through *vpu* AUG. During maturation of the virus particle, cell proteases cleave the gp160 precursor to yield the gp120 surface subunit (SU) and the gp41 transmembrane subunit (TM) (reviewed by: Cullen, 2003; 2009; Bolinger & Boris-Lawrie, 2009).

The lipid-enveloped HIV virion measures approximately 80–120 nm in diameter and contains a number of host cellular proteins important for virus replication and pathogenesis. As many as 253 proteins have been found in HIV-1 virion preparations from infected cells (Chertova *et al.*, 2006; Saphire *et al.*, 2006; for review see: Kolegraff *et al.*, 2006; Ott, 2008). Figure 2A depicts the structural organization of HIV-1 particle.

HIV-1 LIFE CYCLE

The key to understanding the course of HIV infection and the Lentivirus life cycle in general is the unraveling of the virus–host protein interactions. HIV-1 genome, like those of all viruses, is too small to encode all the components necessary for its replication, therefore the virus must "hijack" the host cell machinery in order to complete its life cycle. Recently, the Division of AIDS of the National Institute of Allergy and Infectious Diseases, in collaboration with Southern Research Institute and the National Center for Biotechnology Information (NCBI), has initiated the development of an *HIV-1, Human Protein Interaction Database* (see List of websites [8]). Thirty-two hundred papers published between 1984 and 2007, describing putative interactions between HIV-1 and human proteins were identified by PubMed queries. A total of 1448 human proteins that interact with HIV-1, comprising 2589 unique HIV-1–to–human protein interactions, were found. Two HIV proteins stand out due to their most complex interplay with the host factors: surface glycoprotein gp120 and regulatory protein Tat (532 and 775 interactions, respectively) (Ptak *et al.*, 2008; Fu *et al.*, 2009).

The HIV-1 life cycle has been thoroughly described by others (Freed, 2001; Finzi *et al.*, 2007a; Adamson & Freed, 2007; Spirin *et al.*, 2008; Klimas *et al.*, 2008). Figure 2 summarizes our current knowledge about the HIV-1 replication phases. Here we focus on the crucial virus–host protein–protein interactions and the viral genome landmarks that determine individual steps of viral infection.

Entry

The HIV replication cycle starts (*early phase* of infection) when the viral envelope glycoprotein gp120 binds to the CD4 receptor exposed on the surface of the host CD4-positive cells (Fig. 2A) (Dalgleish *et al.*, 1984; Landau *et al.*, 1988). This binding triggers a series of events that lead to the ultimate destruction of those cells. The gp120–CD4 interactions may contribute to the depletion of CD4+ T cells through an interferon (IFN) α/β-induced, TRAIL (tumor necrosis-related apoptosis-inducing ligand)-mediated apoptotic mechanism (Herbeuval *et al.*, 2005; Herbeuval & Shearer, 2007). Recently, it has also been shown that anti-CD4 antibodies prevent HIV-induced indoleamine 2,3-dioxygenase (IDO) expression in plasmacytoid dendritic cells (pDCs) (Boasso *et al.*, 2007). IDO is an immunosuppressive enzyme that inhibits T cell proliferation. It was proposed that HIV-activated CD4+ pDCs, *via* production of type I IFN and IDO, may exert suppressive and cytotoxic effects on T cells (Boasso & Shearer, 2008; Boasso *et al.*, 2008). Interestingly, gp120 alone, when incubated with human T cells, can affect CD4+ T cell migration. An increased lymph node accumulation of CD4+ T cells observed in HIV+ patients, with concurrent decreases in blood and spleen, could be a reason of HIV-related lymphadenopathy and lymphopenia (Green *et al.*, 2009). Additionally, HIV-1 negative factor Nef inhibits T lymphocytes chemotaxis and diminishes adhesion and polarization of the T cells. Recent results suggest that membrane localization of the Nef and intracellular signaling events might contribute to these inhibitory effects (Park & He, 2009).

After binding to CD4, activated gp120 undergoes a conformational change which produces a binding site for a secondary host cell receptor (Sattentau & Moore, 1991). The most common strains of HIV-1 utilize a β-chemokine receptor, CCR5, or an α-chemokine receptor, CXCR4, as coreceptors (Alkhatib *et al.*, 1996; Feng *et al.*, 1996). HIVs that use only the CCR5 receptor are termed R5, those that only use CXCR4 are termed X4, and those that use both, X4R5. Infections with X4 and R5 strains differ in their effects exerted on host cells. It has been shown recently that R5 viruses modulate, to a greater extent than X4 viruses, the level of mRNA of the resting CD4+ T cells. R5 viruses, but not X4 strains, are able to modulate T cell receptor (TCR)-mediated actin polymerization and signaling. Additionally, a number of genes induced exclusively by R5 envelopes were also up-regulated in the resting CD4+ T cell population, which might facilitate replication of HIV in the pool of resting CD4+ T cells (Cicala *et al.*, 2006; Sir-

ois *et al.,* 2008). R5-infected cells have a higher rate of activation and proliferation (Locher *et al.,* 2005) and R5 viruses produce more progeny in infected CD4+ T cells than do X4 strains (Schweighardt *et al.,* 2004). As other G-protein coupled receptors, CCR5 and CXCR4 have a seven transmembrane structure. The N-terminal portion is extracellular and participates in the binding of the chemokine, whereas the C-terminus is intracellular and interacts with β-arrestin. In addition to binding the chemokine receptor to clathrin for endocytosis and recycling, β-arrestins facilitate G-protein-independent cell signaling resulting in stimulation of protein synthesis (Zhao *et al.,* 1998; Oppermann, 2004; Signoret *et al.,* 2005; DeWire *et al.,* 2008). The association of CD4 with gp120 becomes unstable, once gp120 begins to bind with CCR5, giving a tighter gp120–CCR5/CD4 complex (Chang *et al.,* 2005; Dobrowsky *et al.,* 2008). Identifying CCR5 as the coreceptor for the majority of transmissible strains of HIV has led to the development of entry inhibitors (reviewed by Dhami *et al.,* 2009). Moreover, people homozygous for a 32-bp deletion in this coreceptor gene are genetically resistant to infection by HIV (Liu *et al.,* 1996; recently reviewed by Piacentini *et al.,* 2009).

Formation of the gp120–coreceptor/CD4 complexes triggers refolding of the non-covalently associated transmembrane gp41 protein. It is not clear whether disassociation of gp120 is required for further steps of infection, but there is evidence that the interactions between gp120 and gp41 must weaken in order to initiate fusion (Abrahamyan *et al.,* 2003). Activated gp41 protein exposes an N-terminal hydrophobic fusion peptide inserted into the cellular plasma membrane. The heptad repeat regions (HR1 and HR2) of the gp41 trimer subsequently fold in to form a six-helix bundle (6HB or coiled-coil complex). The formation of this complex couples the viral and cellular membranes (creation of so-called fusion pore) and releases free energy sufficient to promote their fusion (Fig. 2B) (Melikyan *et al.,* 2000; Markosyan *et al.,* 2003; reviewed by Melikyan, 2008).

Once the fusion is initiated, the viral core contents are released into the cytoplasm. It is postulated that HIV-1 Nef induces actin rearrangements, thereby reinforcing the core's movement and penetration of the cortical actin barrier underlying the plasma membrane after fusion (Campbell *et al.,* 2004). This requirement of Nef is avoidable when the genuine HIV Env is replaced (pseudotyped virus) with pH-dependent envelope glycoproteins derived from, for example, vesicular stomatitis virus (VSV-G), which enters cells *via* endocytosis with subsequent endosome acidification releasing the virion core (Chazal *et al.,* 2001). However, a more recent study has shown that the infectivity stimulation by Nef is HIV-1 Env-independent and is rather attributed to virus interactions with cellular receptors (Pizzato *et al.,* 2008). Thus, the Nef involvement in the early stages of HIV-1 entry is still controversial and other recent results call into question its importance during virion release into the cytoplasm, pointing out to its role in the biogenesis of viral particles instead (Laguette *et al.,* 2009).

Despite the fact that the actin cytoskeleton may act as a barrier to incoming viruses at the plasma membrane it also plays a positive role in early retroviral trafficking (Bukrinskaya *et al.,* 1998; Komano *et al.,* 2004). The dependence of lentiviral infection on actin polymerization suggests that the actin network facilitates short-range motion of the viral cores to another region of the peripheral cytoplasm and subsequently links them to microtubules for efficient transport to the nucleus.

The fine mechanism mediating the intracellular transport of the virus capsid is poorly understood and is only known to involve the microtubule system. Live cell observation of viral trafficking, using fluorescently labeled tubulin, allowed visualizing of the movement of individual viral cores along microtubules (McDonald *et al.,* 2002). This trafficking required a minus-end microtubule motor, dynein. Dyneins move cargo toward the minus end of microtubules, which is anchored at the microtubule organizing center (MTOC) or centrosome, in a process that is called retrograde transport (Fig. 2C). In another study, HIV-1 complexes with fluorescently labeled viral integrase showed directed movement toward the nuclear compartment, kinetically characteristic of both microtubule- and actin-dependent transport (Arhel *et al.,* 2006).

Host defense

Viral replication depends on host cell factors and species-specific characteristics of these proteins affect viral tropism. On the other hand, evolution has equipped cells with factors that can directly inhibit retrovirus replication. This cellular anti-viral system, mediated by so-called restriction factors, is termed intrinsic immunity as distinct from conventional innate and adaptive immunity, which engage a number of genes controlling expression of antiviral cytokines (interferons — IFNs) (reviewed by: Akira *et al.,* 2006; Kang & Compans, 2009). To combat the host defense, some HIV-1 auxiliary gene products counteract these host inhibitors and, thus, no human protein can effectively block replication of wild type HIV-1 under physiological conditions. Understanding of this intricate interplay between host defense system, the species-specific barriers, and the viral infectivity factors is key in the ongoing search for new potent antiviral drugs. Recently, high-throughput methods have been used to identify factors that might be involved in antiviral immunity (Valente & Goff, 2009).

Currently, the most intensively studied host restriction proteins are: tripartite motif protein 5α (TRIM5α), cyclophilin A (CypA), apolipoprotein B mRNA-editing catalytic polypeptide 3 (APOBEC3), zinc finger antiviral protein 2 (ZAP2), eukaryotic initiation factor 3 subunit f (eIF3f) (Valente *et al.*, 2009), heterogeneous nuclear ribonucleoprotein U (hnRNP U), and cell surface protein tetherin/CD317. In the context of host defense, the following viral proteins are key players: viral infectivity factor (Vif), viral protein U (Vpu), and viral protein R (Vpr) (recently reviewed by: Towers, 2007; Wolf & Goff, 2008; Takeuchi & Matano, 2008; Huthoff & Towers, 2008; Aguiar & Peterlin, 2008; Goila-Gaur & Strebel, 2008; Malim, 2009). Here, we will describe viral proteins that interact with cellular factors in order to abrogate restriction.

APOBEC3G (catalytic polypeptide 3G or A3G), a member of the family of cellular polynucleotide cytidine deaminases, was first identified as a cellular factor that protects human cells from infection by HIV-1 viruses lacking a *vif* gene (HIV-1Δ*vif*) (Sheehy *et al.*, 2002). A3G together with another family member, A3F, in the absence of Vif are encapsidated by budding virus particles and lead to excessive cytidine (C) to uridine (U) editing of negative sense reverse transcripts in newly infected cells. The C to U editing results in guanine (G) to adenine (A) substitutions in the positive sense DNA strand as reverse transcription is completed (Harris *et al.*, 2003). However, it seems that the main antiviral effects of APOBEC3 proteins are achieved by an editing-independent mechanism: inhibition of viral DNA synthesis by impeding the translocation of reverse transcriptase along template RNA (Anderson & Hope, 2008; Bishop *et al.*, 2008). It is possible that to perform this action A3G/A3F needs another cellular cofactor, since not all cell lines are resistant to HIV-1Δ*vif* virus infection (Han *et al.*, 2008). In physiological conditions, Vif binds to A3G, A3F, and A3C and targets these proteins for polyubiquitination by forming an E3 ubiquitin ligase with cullin 5 (Cul5 — E3) (Yu *et al.*, 2003a; Liu *et al.*, 2005; Kobayashi *et al.*, 2005; Zhang *et al.*, 2008). It is also possible that Vif can inactivate the cellular restriction factors without their degradation (Kao *et al.*, 2007; Ao *et al.*, 2008). The ability of Vif to block the antiviral activity of APOBEC3 is species-specific and this specificity might be a barrier for interspecies virus transfers (Simon *et al.*, 1998; Mariani *et al.*, 2003; Schröfelbauer *et al.*, 2006). Recent identification of the binding sites responsible for this intriguing phenomenon may lead to generation of pharmacologic agents that would inter-

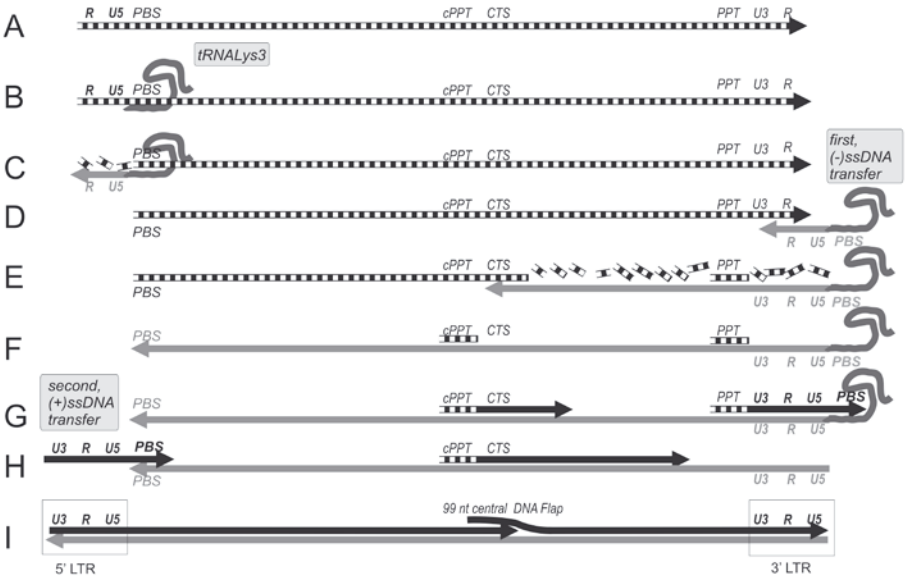

Figure 3. Reverse transcription.
Replication of lentiviral genomic RNA (gRNA) (**A**) is conducted by viral reverse transcriptase (RT). The process starts by binding of tRNALys primer to the primer binding site (PBS) (**B**). The primer is extended until the end of gRNA molecule, and RNA from the RNA/DNA hybrid is digested by RNase H subunit of RT. Resulting (-) single-stranded DNA called strong stop DNA, (-)ssDNA, is transferred to the 3′ end of gRNA and annealed *via* R region in a process called first (-) strong stop DNA transfer (**D**). This initiates the synthesis of the entire (-) DNA strand of the provirus (**E**). Simultaneous digestion of gRNA spares polypurine tracks, central polypurine track (cPPT) and 3′ polypurine track (PPT) (**F**) which in turn serve as primers for (+) DNA strand synthesis (**G**). When the 3′ LTR, together with PBS region, is transcribed from PPT primer, the second (+) strong stop DNA transfer occurs. The created (+) single stranded DNA fragment is annealed to the 5′ (-) DNA strand *via* PBS region (**H**). This enables reconstruction of 5′ LTR and synthesis of (+) DNA strand up to the center of the genome. In the CTS region DNA synthesis is ended and central DNA Flap is formed (**I**). Dotted line represents gRNA; grey line: (-) DNA strand; black line: (+) DNA strand. Arrows indicate 5′→3′ orientation and direction of DNA synthesis.

fere with the interactions between Vif and APOBEC3 proteins and bring about efficient inhibition of viral replication (He *et al.*, 2008; Pery *et al.*, 2009).

HIV-1, like other RNA viruses, has also developed a specific mechanism to downregulate the early antiviral response. It has been shown that viral accessory proteins Vif and Vpr can independently target an interferon regulatory factor, IRF-3, for ubiquitin-mediated degradation (Okumura *et al.*, 2008).

Another example of how lentiviruses counteract the innate host defense is the blocking of activation of the interferon-induced serine/threonine protein kinase R (PKR) pathway by the viral protein Tat. Tat interferes with autophosporylation of PKR or acts as a competing substrate (reviewed by Bolinger & Boris-Lawrie, 2009). It has also been demonstrated recently that Tat impairs the interferon gamma (IFNγ)-receptor signaling pathway by inducing the activity of suppressor of cytokine signaling-2 (SOCS-2) (Cheng *et al.*, 2009).

Recently, an inhibitor of HIV-1 particle release termed tetherin (CD317), whose activity is neutralized by the HIV-1 accessory protein Vpu, has been discovered (Neil *et al.*, 2008). CD317, an interferon-induced membrane protein, causes retention of virions on the cell surface and, after endocytosis, in CD317-positive compartments. Vpu co-localizes with this protein and inhibits these effects. Interestingly, Vpu activity is species specific and HIV-1 Vpu protein has evolved to target only the transmembrane domains of human and chimpanzee tetherins (McNatt *et al.*, 2009).

Reverse transcription

In the cytoplasm, virions undergo structural alterations giving rise to a large ribonucleoprotein structure called the reverse transcription complex (RTC), the site of extensive viral DNA synthesis (Fig. 2C). *In vitro* studies have shown that viral RNA is partly released from the core upon reverse transcription initiation and is used as a template for the RT to synthesize provirus DNA (Auewarakul *et al.*, 2005). Yet *in vivo*, this synthesis takes place rather in an intact capsid (Arhel *et al.*, 2007).

Reverse transcription requires a specific cellular tRNA annealed to the primer binding site (PBS) for the initiation of cDNA synthesis. In the case of HIV-1, tRNALys3 is preferentially selected during virus assembly. It is incorporated into the virion *via* interactions of the RT domain of Gag-pol with Gag and lysyl-tRNA synthetase (LysRS) (Barat *et al.*, 1989; Saadatmand *et al.*, 2008; Bilbille *et al.*, 2009). After annealing of the primer and cDNA synthesis initiation, reverse transcription involves two obligatory DNA strand transfers to carry out the synthesis of the complete double-stranded proviral DNA (dsDNA) with duplicated long terminal repeat (LTR) ends (Fig. 3). Transcription of the plus strand RNA to minus strand DNA requires a transfer of the growing DNA strand from one end of the genomic RNA (gRNA) to the other. This step is called "minus strong-stop DNA" transfer. Subsequent synthesis of the second (plus) DNA strand involves priming from cPPT and 3′ PPT followed by the second strand transfer. Synthesis of the plus DNA strand continues until it reaches CTS, which leads to the creation of central DNA Flap. A basic feature of the strand transfer mechanism is the use of RNase H, another activity of RT, to remove fragments of the RNA template from the growing DNA strand (with the exception of the polypurine tracts of cPPT and PPT), enabling a single stranded DNA region to anneal to this second site (reviewed by Basu *et al.*, 2008). These processes are accompanied by nucleocapsid protein, NC, a potent nucleic acid chaperone, which tightly binds nucleic acids and facilitates the annealing of complementary sequences as well as the strand transfer and exchange reactions (Guo *et al.*, 2000; reviewed by: Basu *et al.*, 2008; Mougel *et al.*, 2009).

The nascent viral DNA binds several viral and cellular proteins to produce so-called preintegration complex (PIC) (Fig. 2D).

Nuclear import

As mentioned before, lentiviruses, unlike simple retroviruses, are able to infect nondividing cells. This intriguing phenomenon can be explained by the fact that lentiviral PICs with the size of 56 nm (Miller *et al.*, 1997) are actively transported through nuclear pore complexes (NPCs) in an ATP-dependent manner (Bukrinsky *et al.*, 1992). As shown earlier, cargoes much larger than the assumed functional NPC diameter of 26 nm must be imported into the karyoplasm *via* an active transport mechanism (Panté & Kann, 2002). Both viral and cellular elements take part in this process. In contrast to the HIV-1 PIC, during infection of the dividing cells by a simple Moloney murine leukemia virus (MLV) the reverse transcriptase complex/preintegration complex (RTC/PIC) retained a significant portion of capsid protein (CA) (Fassati & Goff, 1999). Moreover, the MLV CA appears to be the dominant negative regulator of nuclear import (Yamashita & Emerman, 2004). Since HIV-1 mutants that do not shed enough p24 CA are defective in nuclear import and integration, it has been proposed that appropriate shedding of CA protein from the lentiviral RTC/PIC is a key step for the RTC-to-PIC transition and subsequent nuclear import of HIV-1 (Dismuke & Aiken, 2006).

According to this view, blocks in reverse transcription and in RTC maturation might be one of the reasons why lentiviruses cannot infect naïve T

lymphocytes *in vitro* (Zack *et al.*, 1990; Auewarakul *et al.*, 2005). It was recently shown, that CA-containing sub-viral complexes accumulated at centrosomes of human primary quiescent CD4+ T cells, were stably persisting there for weeks and could be induced to replicate upon cell activation. This might reflect strategy that has evolved for the virus to persist latently in the vicinity of this organelle in resting cells and to be activated in response to cell activating stimuli (Zamborlini *et al.*, 2007). It has long been known that in AIDS patients quiescent T lymphocytes constitute an inducible reservoir of latent HIV-1 viruses (Bukrinsky *et al.*, 1991). Yet more recently, it was convincingly demonstrated that HIV-1 could replicate in these cells *in vivo* (Eckstein *et al.*, 2001) and that also *in vitro* a tiny fraction of the virus population could integrate into the host genome in sites similar to those selected in prestimulated cells (Vatakis *et al.*, 2009). However, the integration process in quiescent T cells is hindered by the nuclear accumulation of large numbers of incorrectly processed viral ends and abortive single- and double-LTR (1- and 2-LTR) circles that are common dead-end by-products of viral replication.

Mature HIV-1 PICs contain provirus DNA and proteins such as reverse transcriptase (RT), integrase (IN), nucleocapsid (NC), viral protein R (Vpr), and matrix (MA) (Fig. 2D) (Bukrinsky *et al.*, 1993a), although the presence of the NC has been questioned (Miller *et al.*, 1997) and disassociation of RT, once cDNA synthesis in RTC is completed has been postulated (Bukrinsky, 2004). MA and IN have functional nuclear localization sequences (NLSs) that resemble the canonical NLS of the simian virus 40 (SV40) T antigen and both utilize the classical nuclear import pathway dependent on importins α and β (karyopherins) (Bukrinsky *et al.*, 1993b; Gallay *et al.*, 1996; 1997). HIV-1 also exploits importin 7, a member of importin β family, to maximize nuclear import of its PICs *via* interaction with IN (Ao *et al.*, 2007). However, this is not true for the HIV-2, SIVmac or EIAV integrases. This suggests that the closely related lentiviruses may have evolved different and probably redundant nuclear import mechanisms (Zaitseva *et al.*, 2009).

In another recent study, HIV-1 IN was shown to directly interact with NPC *via* binding to nucleoporin NUP153. Overexpression of NUP153 C-terminal domain in cultured cells reduced the infectivity of an HIV-based vector by interfering with the nuclear import of the viral PIC (Woodward *et al.*, 2009).

Also the Vpr C-terminus contains several arginine residues that resemble a basic NLS, but this sequence does not function as an NLS. Nonetheless, it was shown that Vpr associates with importin α, and that this association results in an increased binding affinity of the basic-type NLSs, including that of MA (Popov *et al.*, 1998; Nitahara-Kasahara *et al.*, 2007).

An additional viral element implicated in HIV-1 nuclear import is the central polypurine tract (cPPT). Located within *pol* gene the cPPT is the second origin of DNA plus strand synthesis that, after completion of reverse transcription, results in a short, approximately 100 nt-long, stretch of triple-stranded DNA (so-called central DNA Flap) (Charneau *et al.*, 1992) (Fig. 3; for details see section on Reverse Transcription). When cPPT is mutated, Flap-defective linear DNA accumulates in close proximity of the nuclear membrane, indicating a late defect in nuclear import (Zennou *et al.*, 2000). On the other hand, insertion of the cPPT into HIV-based vectors strongly stimulates gene transfer in target cells (for references see section on cPPT–CTS below). The mechanism of action employed by this *cis*-acting element was unravelled by Charneau's group. They showed that maturation of RTC into PIC, followed by uncoating of the preintegration complex at the nuclear pore, is impaired in the absence of DNA Flap formation, consequently leading to the trapping of HIV-1 PIC within an integral capsid shell and subsequently prohibiting nuclear entry of the viral genome (Arhel *et al.*, 2007). According to those observations, reverse transcription proceeds within an intact CA shell, independently of the RTC movement toward the nuclear membrane. Hence, *in vivo*, uncoating does not occur immediately after virus entry and reverse transcription initiation (as was observed *in vitro*: Auewarakul *et al.*, 2005), but starts at the nuclear pore upon completion of cDNA synthesis and Flap forma-

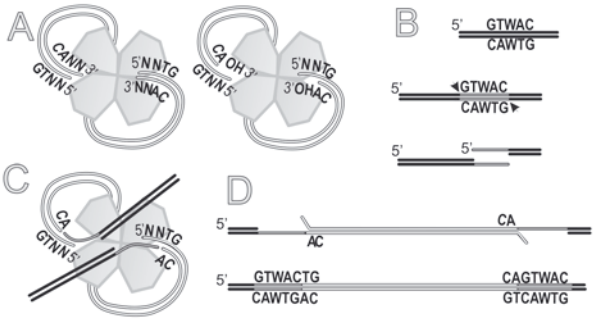

Figure 4. Scheme of provirus integration.
Integration of provirus to the host genome DNA is performed by lentiviral integrase (IN). The enzyme recognizes 5′ and 3′ short terminal fragments called *att*, binds and bends the DNA molecule, removes two nucleotides from each 3′ end (staggered cut) and exposes OH group (*3′ processing*) (**A**). IN cleaves host genomic DNA, creating five-nucleotide-long overhangs (**B**), which then are bound to the free 3′ OH groups of the provirus (*DNA strand transfer*) (**C**). The last step of integration comprises DNA repair of five-nucleotide gaps at both sides of proviral DNA by cellular enzymes (**D**). IN is shown in tetrameric form. GTWAC–HIV-1 target site consensus sequence (W means A or T).

tion (Fig. 2D) (Arhel *et al.,* 2007). It was recently postulated that the additional initiation of plus strand DNA synthesis from the cPPT RNA primer facilitates the efficient completion of proviral DNA synthesis in viruses harboring kinetically impaired RT, because the enzyme needs only to synthesize half of the genome during plus strand synthesis (Skasko & Kim, 2008).

Also other factors might be involved in the HIV-1 DNA entry to the nucleus, e.g., tRNA (Zaitseva *et al.,* 2006). On the other hand, numerous results concerning this issue are controversial or conflicting. For example, participation of the MA and IN proteins in HIV-1 genome nuclear import, as well as implication of the central DNA Flap in this process, are still a matter of debate (Freed *et al.,* 1997; Limon *et al.,* 2002a; 2002b; Dvorin *et al.,* 2002). Thus, the mechanisms of lentiviral PIC nuclear import, a critical step in the virus life cycle, need to be further investigated.

Integration

In the nucleus, the linear provirus cDNA integrates into the DNA of the host cell (Fig. 2E). Viral integrase (IN) circularizes the provirus at the *att* repeats and plays a crucial role in its integration into host cell DNA *via* recombination between the *att* repeats and the integration site (Fig. 4). It was shown that IN is the principal viral determinant of integration specificity (Lewinski *et al.,* 2006). Wu *et al.* (2005) and Holman and Coffin (2005) have examined a large number of integration site sequences from various retroviruses, including HIV-1, and found that they are palindromic in nature. The symmetry of the target site sequence is a consequence of the fact that the IN forms symmetrical dimers, tetramers, or oligomers and that each half complex would have a similar preference for target DNA structure.

IN catalyzes two spatially and temporally distinct reactions. During the first reaction, called 3′ processing, IN hydrolyzes two nucleotides from both 3′ ends of HIV-1 DNA. The second reaction, DNA strand transfer, takes place at the site of integration. IN uses the recessed 3′-OH groups created during 3′ processing to cleave the phosphodiester backbone of chromosomal DNA in a staggered fashion, concomitantly connecting the viral DNA 3′ ends to the generated 5′ overhangs by transesterification mechanism. The resultant DNA recombination intermediate harbors single-strand discontinuities and the process is completed by the third main step, gap repair by host cell enzymes. As a result of the integration a duplication of 5 bp-long fragment of host DNA flanking the provirus is created. The consensus sequence at the HIV-1 palindromic duplicated target sites is $G_1T_2(A/T)_3A_4C_5$ (Wu *et al.,* 2005; Holman & Coffin, 2005) (for recent reviews on HIV-1 integrase see: Vandegraaff & Engelman, 2007; Delelis *et al.,* 2008; Poeschla, 2008).

Although purified retroviral IN enzyme displays 3′ processing and DNA strand transfer activities *in vitro* (Bushman *et al.,* 1990; Li & Craigie, 2009), *in vivo* it acts in the context of PIC and numerous studies indicate that other proteins play important auxiliary roles in modulation of its activity. In HIV-infected cells, the regulator of viral protein expression Rev may be involved in the integration of proviral DNA by regulating the activity of the integrase. It has recently been shown that these two proteins interact with each other *in vitro* and *in vivo* and that Rev-derived peptides can inhibit the 3′-end processing and strand-transfer enzymatic activities of IN *in vitro* (Rosenbluh *et al.,* 2007). On the other hand, IN-derived peptides exert an opposite effect stimulating IN activity and multiple viral DNA integration events due to displacement of the Rev-derived inhibitory molecules (Levin *et al.,* 2009). This suggests that lentiviruses have evolved mechanisms to limit the frequency of integration events in the infected cell. Transcriptionally active unintegrated HIV-1 genomes present in the infected cells can participate in HIV-1 replication and contribute to its genetic diversification and evolution (Gelderblom *et al.,* 2008).

Among the cellular proteins that have been shown to affect lentiviral integration, recent attention has focused on LEDGF/p75 (Maertens *et al.,* 2003). LEDGF/p75 and HRP2, members of the hepatoma-derived growth factor (HDGF)-related protein (HRP) family, bind directly to IN within the viral PIC and this interaction is exclusive to lentiviruses (Llano *et al.,* 2004; Busschots *et al.,* 2005; Cherepanov, 2007). LEDGF/p75 is a ubiquitous nuclear protein, tightly associated with chromatin through three conserved sequence elements within the N-terminal half of the protein (N-terminal domain ensemble, NDE): the PWWP domain, NLS, and a dual copy of the AT-hook DNA binding motif (Llano *et al.,* 2006; Shun *et al.,* 2008). The C-terminal integrase-binding domain (IBD) interacts with the viral IN catalytic core domain (CCD) (Cherepanov *et al.,* 2004; Busschots *et al.,* 2007; Hombrouck *et al.,* 2007). The cellular functions of LEDGF/p75 are probably involved in the survival response to environmental stress, and alternatively spliced LEDGF gene (*PSIP1*) products yield two coactivators of transcription: p75 and p52 (Ge *et al.,* 1998; Singh *et al.,* 2000; Nishizawa *et al.,* 2001). For lentiviruses, LEDGF/p75 serves as an infectivity factor and a key determinant of the preferential integration target site selection within transcriptional units, likely acting as a receptor or molecular tether (Fig. 2E) (Ciuffi *et al.,* 2005; Shun *et al.,* 2007; Marshall *et al.,* 2007; Hombrouck *et al.,* 2007). However, some recent results suggest that chromatin tethering

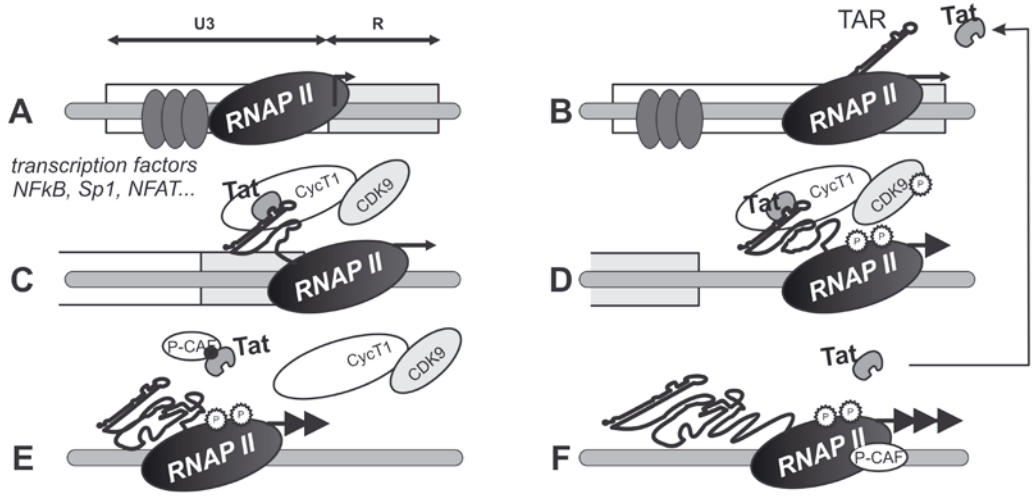

Figure 5. HIV-1 transcription initiation and elongation.
U3 region of 5′ LTR contains sites recognized by various cellular transcription factors. RNAP II-directed transcription starts at the first nucleotide of R region with low efficiency (**A**). Binding of viral Tat transactivation protein to nascent transcript in TAR region (**B**) enhances polymerase activity *via* recruiting elongation factor composed of cyclin CycT1 and kinase CDK9 (**C**). CDK9 phosphorylates RNAP II, which result in dramatic stimulation of its processivity (**D**). Acetylation of Tat by P-CAF liberates it from TAR (**E**) and facilitates RNAP II–P-CAF association to further stimulate transcription elongation (**F**).
Abbreviations: U3, 3′ unique element; R, repeat element; RNAP II, RNA polymerase II; TAR, transactivation response element.

per se rather than association with specific chromatin ligands is central to the LEDGF/p75 mechanism (Meehan *et al.,* 2009). It has been shown that binding to either DNA or protein molecules in chromatin as well as docking either within or outside the nucleosome is sufficient for the activity of LEDGF/p75 as an HIV-1 cofactor. According to these observations, while the basic LEDGF/p75 role is fulfilled without NDE, NDE domains might play more subtle roles in viral replication and selection of integration sites.

The importance of this protein for Lentivirus infectivity is not limited to the functions presented above and can be expanded by the following activities: enhancement of the nuclear import of PIC, stimulation of IN strand transfer and protection of IN protein from proteasomal degradation (for review see: Poeschla, 2008).

HIV-1 PICs also interact with other cellular proteins important for performing the PICs functions: integrase interactor 1 (INI-1/hSNF5), the first identified IN cofactor, one of the core subunits of the ATP-dependent chromatin remodeling complex SWI/SNF that regulates expression of eukaryotic genes by modifying DNA/histone interactions (Kalpana *et al.,* 1994); high mobility group protein A1 (HMGA1, formerly HMG I/Y), a non-histone chromosomal factor involved in transcriptional control and chromosomal architecture (Farnet & Bushman, 1997; Miller *et al.,* 1997; Hindmarsh *et al.,* 1999); Ku70/Ku80 heterodimer, a component of the non-homologous DNA end joining (NHEJ) pathway engaged in double-stranded gap repair (Li *et al.,* 2001); human heat shock protein 60 (hHSP60), a protein chaperone (Parissi *et al.,* 2001); barrier-to-autointegration factor (BAF), a protein highly conserved in metazoa with structural roles linking chromatin organization, nuclear architecture and gene regulation (Lin & Engelman, 2003; Mansharamani *et al.,* 2003); and TNPO3/transportin-SR2 (TRN-SR2), a nuclear import receptor for serine/arginine-rich (SR) proteins, important splicing regulators (Christ *et al.,* 2008; Brass *et al.,* 2008).

Gene expression

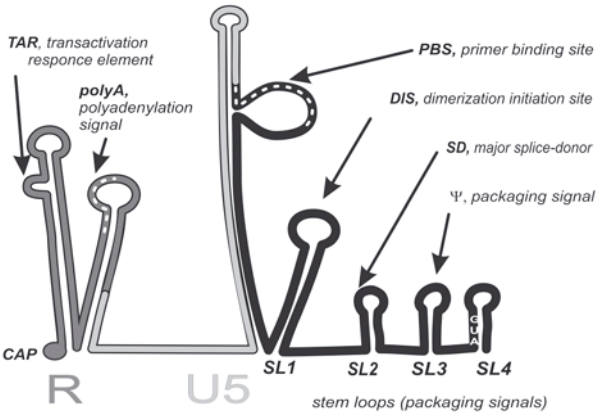

Figure 6. Schematic representation of RNA structure of HIV-1 5′ UTR and 5′ end of *gag* gene.
The 5′ end of viral mRNA is capped. The *gag* AUG start codon is highlighted. Dark-grey line shows R region, light-grey line — U5 region. Dotted white lines depict positions of polyA and PBS sites on stem loops. RNA elements important for HIV-1 life cycle, including stem loops (SL) 1–4, are indicated.

The *late phase* of the lentiviral life cycle starts when proviral DNA is transcribed by cellular RNA polymerase II (RNAP II) (Fig. 2F). HIV exploits the cellular transcriptional machinery in a sophisticated way. First, before the integration step is completed, the infected cell is arrested in the G2 phase of the cell cycle. This favors viral replication because the promoter elements present in the LTR seem to be most active in G2 (Goh *et al.,* 1998). The block in the cell cycle progression is mediated by Vpr *via* indirect inactivation of the Cdc2 (CDK1)/cyclin B (CycB) kinase and Cdc25 phosphatase. These are regulators of the G2/M transition, possibly activated by the ataxia telangiectasia-mutated and Rad3-related (ATR) DNA damage response pathway (reviewed by: Amini *et al.,* 2004; Le Rouzic & Benichou, 2005; Andersen *et al.,* 2008). However, there is also evidence that in the case of other lentiviruses (i.e., African green monkey SIV, SIVagm) the transactivation of LTR by Vpr is, in part, G2 arrest-independent (Zhu *et al.,* 2001a).

Second, for initiation of its transcription, HIV usurps distinct cellular signaling pathways by using nuclear factor kappa-B (NF-κB) and nuclear factor of activated T cells (NFAT) (reviewed by: Cullen, 1991; Pessler & Cron, 2004). After T cell activation by TCR, downstream from phospholipase C (PLC), NF-κB is activated *via* the diacylglycerol/Ca^{2+}/protein kinase C (PKC) pathway, whereas NFAT is activated by the IP3/Ca^{2+}/calcineurin pathway (for review see: Tan & Parker, 2003). NF-κB and NFAT binding sites along with binding sites for other cell-specific and constitutively expressed cellular transcription factors are located in the U3 enhancer/promoter region of 5′ LTR, upstream from the TATA box (Fig. 5).

The Tat protein is the main Lentivirus-encoded transcription transactivator. Tat interacts with the *cis* elements of the virus located in the junction between the U3 and R regions of the proviral 5′ LTR, to increase *the processivity of* RNAP II. Unlike typical transcriptional transactivators, Tat binds not to DNA, but to an RNA bulge of a stem-loop structure present at the 5′ end of all HIV-1 transcripts, known as the transactivation response element (TAR) (Fig. 6) (Feng & Holland, 1988). Thus, Tat requires the initial transcription of TAR before it can stimulate further HIV transcription (for review see: Cullen, 1991; Brady & Kashanchi, 2005; Barboric & Peterlin, 2005). The basal transcriptional activity from the HIV LTR is very low and RNA synthesis is greatly increased (by more than two logs) when Tat is present (Fisher *et al.,* 1986; Kao *et al.,* 1987). Tat binds positive transcription elongation factor (P-TEFb), an elongation factor composed of cyclin T1 (CycT1) and kinase CDK9, and recruits it to TAR (Zhu *et al.,* 1997; Wei *et al.,* 1998). Subsequently, CDK9 phosphorylates the carboxy-terminal domain (CTD) of RNAP II, which in turn results in dramatic stimulation of transcriptional processivity. The strength of the Tat-P–TEFb association is adjusted by autophosporylation of CDK9-stronger association (Garber *et al.,* 2000) and acetylation of Tat-weaker association (Kiernan *et al.,* 1999). This acetylation is mediated by p300/CREB-binding protein (CBP)-associated factor (P-CAF). Free acetylated Tat protein is now able to recruit P-CAF to phosphorylated RNAP II to further stimulate transcript elongation (Fig. 5). Acetylation of Tat is used to modulate the strength of the viral–host RNA–protein complex, thereby fine-tuning the efficiency of transcription elongation. It has recently been reported that in contrast to HIV-1 Tat, the bovine Lentivirus transactivator has evolved a high-affinity TAR interaction that does not require P-CAF-mediated acetylation. Additionally, HIV-2 Tat exploits an intermediate mechanism that uses a duplicated TAR element and CycT1 to enhance RNA binding (D'Orso & Frankel, 2009).

Other results link the Tat–TAR axis to the latency, which is typical for Lentivirus infection. In HIV-1-infected resting CD4+ T cells, accumulation of Tat variants with impaired transactivation activity was observed, suggesting that impaired Tat activity may contribute to mechanisms responsible for latent infection with HIV (Yukl *et al.,* 2009). In experiments involving Jurkat T cells infected with the HIV vector expressing Tat in *cis*, gene expression was progressively silenced as a result of the formation of restrictive chromatin structures at the viral LTRs (Pearson *et al.,* 2008). This selectively impaired HIV transcription initiation and consequently decreased Tat production below the levels that are required to sustain HIV gene expression. This epigenetic mechanism of HIV transcriptional silencing might allow the virus to enter into latency.

Transcription of viral RNA begins at the first nucleotide of the R region in the 5′ LTR and polyadenylation occurs at the last nucleotide of R in the 3′ LTR. HIV-1 uses the cellular splicing machinery to express its genes (Fig. 2F). Cellular factors, in concert with both positive and negative *cis* elements within the viral genome, act to promote or repress splicing (Jablonski & Caputi, 2009; reviewed by Stoltzfus & Madsen, 2006). These *cis* elements, besides several splice donors (SD) and acceptors (SA), include: three exonic splicing enhancers (ESE), five exonic and intronic splicing silencers (ESS and ISS), and the guanosine-adenosine-rich exonic splicing enhancer (GAR ESE) that seems to be the key player in regulating the creation of both completely spliced and Rev-dependent *vpu/env* transcripts (Asang *et al.,* 2008). Among the cellular factors, members of the SR family of phosphoproteins bind the enhancer elements and promote the use of splice sites, while members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of splicing factors bind the silencer el-

ements and inhibit splice site utilization. The levels of these two classes of cellular splicing factors are regulated during HIV infection thereby affecting virus replication. Changes in expression and, possibly, localization of some splicing factors that modulate viral alternative splicing correlate with Tat cellular levels and virus production in macrophages. Reduced Tat expression and subsequent decline in virus production may contribute to the ability of HIV-1 to persist in the macrophage reservoir (Dowling *et al.,* 2008).

In addition to full-length genomic mRNA (about 9.2 kb in length), coding for the Gag and Gag-pol polyproteins, a number of various subgenomic species of mRNAs have been described in HIV-1-infected cells, with several slightly different mRNAs coding for the same protein (Schwartz *et al.,* 1990; 1991; Purcell & Martin, 1993; Swanson & Stoltzfus, 1998). Of these, two classes of differentially spliced mRNAs can be detected in the cytoplasm: early, fully spliced (about 1.8 kb) and late, partially spliced transcripts (about 4 kb). The former encode the viral regulatory proteins Tat and Rev, along with the auxiliary protein Nef; the latter, accessory proteins: Vif, Vpu, and Vpr as well as the envelope protein Env (Fig. 1B).

Under physiological conditions eukaryotic cells block the nuclear export of unspliced mRNAs (recently reviewed by: Rougemaille *et al.,* 2008; Schmid & Jensen, 2008). Since lentiviruses exploit a variety of differentially and incompletely spliced transcripts, including unspliced genomic RNA, for expression of the complete repertoire of their proteins, they have developed mechanisms to escape this tight export control. The key protein in this process is the regulator of expression of viral proteins, Rev. Rev contains both a nuclear localization signal (NLS) and a nuclear export signal (NES), hence it shuttles between the nucleus and the cytoplasm (Fig. 2G) (reviewed by Pollard & Malim, 1998). Rev, produced from early transcripts, accumulates in the cell and binds to the RNA Rev response element (RRE), a large (roughly 250 nucleotides in size) highly structured RNA element that is located in the *env* gene and is present in all unspliced and partially spliced HIV-1 RNAs (Malim *et al.,* 1989; Pond *et al.,* 2009). RRE constitutes a scaffold for Rev multimerization and the nascent Rev–RRE complex, *via* NES leucine-rich domains, directly binds CRM1, a member of the karyopherin family of nucleocytoplasmic transporters (Fornerod *et al.,* 1997; Yi *et al.,* 2002). This association is stimulated by Ras-related nuclear protein (Ran)/GTP bound to CRM1. In turn, CRM1 interacts with components of the nuclear pore complex (NPC), nucleoporin CAN/Nup214, and this interaction is essential for CRM1-mediated nuclear RNA export (Fig. 2F). In this way HIV "hijacks" the cellular pathway that is used to export small RNAs such as U snRNAs and rRNAs, preribosomal subunits, as well as proteins (reviewed by Cullen, 2009).

The synthesis of viral proteins is regulated by an interplay between host factors and *cis*-acting elements present in viral transcripts (Fig. 1). For example, a weak Kozak sequence surrounding the HIV-1 *vpu* AUG start codon promotes translation of the downstream *env* gene from bicistronic *vpu/env* transcripts, a process referred to as leaky scanning (Schwartz *et al.,* 1992). However, more recent study unexpectedly showed that the different 5′ untranslated regions (UTRs) present in alternatively spliced HIV-1 *vpu/env* mRNA isoforms affected Vpu synthesis, but not the downstream Env synthesis. Therefore, HIV-1 Env may also use an internal ribosome entry sequence (IRES) or the ribosome shunt translation mechanism to maximize Env production (Anderson *et al.,* 2007). Although utilization of IRES to express genetic information *via* a cap-independent mechanism in *Retroviridae* is controversial, the presence of putative internal entry sites has been reported for various lentiviruses (Ohlmann *et al.,* 2000; Buck *et al.,* 2001; Brasey *et al.,* 2003; Herbreteau *et al.,* 2005).

The sequences coding for the Gag and Pol proteins, translated from a full-length unspliced RNA, are in different reading frames. HIV-1 requires a single –1 frameshift to produce the Gag-pol polyprotein. The frameshifting occurs at an approximate rate of one Gag-pol for every twenty Gag molecules synthesized (Jacks *et al.,* 1988). The signal for programmed frameshifting is provided by viral sequences including an upstream slippery sequence. For HIV-1 this sequence is AAUUUUUU (Watts *et al.,* 2009) or UUUUUUA (according to earlier models). Another sequence engaged in the frameshifting is an RNA stem structure located downstream of the frameshift site. Interaction of tRNA with the ribosome, including not only the P and A sites, but also the E site, was postulated to be involved in this process (reviewed by: Bolinger & Boris-Lawrie, 2009; Giedroc & Cornish, 2009).

In addition to the translational mechanisms described above, retroviruses also employ cap-dependent translation enhancers. Post-transcriptional control elements (PCEs), located in the 5′ UTRs, have been found in many viruses, including HIV-1 (reviewed by Bolinger & Boris-Lawrie, 2009). PCEs are stem-loop RNA structures that specifically interact with RNA helicase A (RHA) and facilitate polysome loading and efficient viral protein production. Experiments involving the HIV-1 provirus and the PCE-HIV *gag* reporter RNA indicated that RHA downregulation diminishes HIV-1 *gag* translation independently of the Rev/RRE interactions or of global protein or RNA synthesis.

Also other cellular proteins can bind viral RNA and affect its translation. A direct interaction between TAR and TAR–RNA binding protein (TRBP) enhances synthesis of viral proteins (Dorin *et al.,* 2003). Downregulation of TRBP with small interfering RNAs (siRNAs) decreases basal expression from HIV-1 LTR two-fold and Tat-transactivated level up to ten-fold. Taken together, siRNAs against TRBP decreased the expression of viral genes and inhibited viral production up to five-fold (Christensen *et al.,* 2007).

By contrast, hnRNP E1 exerts an opposite effect on HIV-1 translation. hnRNP E1 binds an HIV-1 exonic splicing silencer present in the bipartite *tat/rev* exon (ESS3a), although this interaction is not associated with a significant changes in viral RNA splicing. Instead, elevated levels of hnRNP E1 decreased Gag (Pr55 and p24), Env (gp160/gp120), and Rev synthesis (Woolaway *et al.,* 2007).

Furthermore, analysis of the RNA structure of the entire HIV-1 genome has led to a number of intriguing findings (Watts *et al.,* 2009). The reported correlation between the organization of the RNA and sequences encoding inter-domain loops in HIV proteins suggests that the RNA structure modulates the speed of ribosome during elongation in order to promote proper protein folding. Thus, differences in RNA sequence accessibility represent additional elements to control the structure and function of HIV proteins.

Assembly and budding

Production of HIV-1 particles by infected cell comprises a series of coordinated events: Gag dimerization and multimerization, binding of Gag complexes to genomic viral RNA (gRNA), transport of the Gag–RNA complexes, Gag-pol, Gag and Env to the site of assembly, and subsequent release of immature virions which later undergo maturation mediated by viral protease to become fully infectious (Fig. 2H–K).

The Gag precursor protein, Pr55, is responsible to balance translation of the full-length primary transcript (pre-mRNA) and its encapsidation (Anderson & Lever, 2006). It has been shown that the Gag polyprotein has a bimodal effect on translation that depends on its concentration: stimulation at low and inhibition at high concentration. This transactivation of translation is presumably mediated by the MA domain of Gag, whereas inhibition results from an interaction between the NC domain and its binding site, packaging/dimerization signal (ψ), in the HIV-1 5′ UTR (Fig. 6). The proposed model assumes that Gag promotes its own synthesis until total covering of the HIV-1 5′ UTR with nascent Gag molecules blocks further translation and directs this RNA to encapsidation. Yet, translation is not prerequisite for unspliced HIV-1 RNA to be directed for packaging into virions and pre-mRNA can function interchangeably as mRNA and as virion precursor RNA (vpRNA) (Fig. 2G,H) (Butsch & Boris-Lawrie, 2000).

In contrast, HIV-2 unspliced RNA is packaged predominantly co-translationally. This discrepancy in the mechanisms used by lentiviruses to ensure that only full-length RNA is packaged can be explained by the differences in the position of ψ with respect to the major splice donor (SD). Unlike in HIV-1, the HIV-2 packaging/dimerization signal is located upstream from the 5′ SD. According to this observation, both HIV-2 RNA species, spliced and unspliced, contain the packaging signal. Thus, the biding of nascent Gag polyprotein to its own template provides a signal for encapsidation of unspliced RNA exclusively (Kaye & Lever, 1999). However, further studies have revealed that *trans*-acting encapsidation of HIV-2 vector RNA is possible in a situation when ψ region-mutated RNA is unable to efficiently capture newly made Gag. The excess of Gag protein is therefore available in *trans* to other RNAs, including spliced species of RNA, that contain an intact packaging signal. This and other results suggest that HIV-1 is able to produce Gag in larger quantities than HIV-2 and it can bind to RNAs anywhere in the cytosol, whereas putative HIV-2 Gag signals might direct it to specific subcellular compartments (Griffin *et al.,* 2001).

While all secondary structures in HIV-1 5′ UTR together with the major packaging signal are important for efficient RNA encapsidation, stem loop 1 (SL1) contains dimerization initiation site, DIS (or dimer-linkage structure, DLS) (Fig. 6) (reviewed by Russell *et al.,* 2004). This raises the question if dimerization of RNA is a prerequisite for its packaging. If this is the case, the dimerization could function as a molecular switch that negatively regulates translation and promotes encapsidation. However, DIS is located upstream of SD (SL2) and thus is present in all spliced RNAs as well. On the other hand, the dimerization signal extends into the 5′ portion of *gag* gene (SL4), and some of its elements lie downstream of the major splice donor. This would provide a convenient mechanism by which only unspliced RNA would be able to dimerize and subsequently become encapsidated. Generally, the encapsidation and dimerization signals overlap in viral genomic leaders (Fig. 6) and the mechanism linking dimerization and packaging of HIV gRNA is still poorly understood. Nonetheless, the packaging of the genome requires specific recognition of dimeric, full-length viral RNA transcript by the NC domain of Gag (Fig. 2H).

An example of the MLV gRNA dimerization mechanism provides valuable insight into this problem. It was observed that MLV RNA dimerization

triggers a conformational change within the packaging signal exposing elements that bind NC with high affinity and thus promote packaging of the diploid genome (D'Souza & Summers, 2004). Consistently with this observation, it has recently been shown that although HIV virion packages two RNA molecules, it takes time to form fully matured dimers and this process requires viral protein maturation (Song *et al.*, 2007). The authors propose a model in which the Gag and Gag-pol polyproteins assemble into a nascent virion containing two gRNA monomers or a fragile dimer. This requires the NC domain of Gag, which may recognize the fragile dimers better than monomers. At this step, DIS is unexposed. Following proteolytic maturation of the viral polyproteins, which is initiated only after the virion buds, products of this maturation (mainly the p9 intermediate and mature p7 NC) switch the DIS from a buried to an exposed conformation. During the first 30 min after virion release, all monomers (fragile dimers) become DIS–DIS "kissing" dimers. After the following several hours, the increasing number of released NC molecules stabilizes full RNA–RNA contacts. Additionally, not only the protease activity and NC maturation appear to be crucial for gRNA dimerization (Kafaie *et al.*, 2008), but also the ratio of the Gag/Gag-pol proteins in the nascent virion as well as CA mutations can impair this process (Shehu-Xhilaga *et al.*, 2001; Kafaie *et al.*, 2009).

Viral structural proteins and the newly synthesized viral RNA molecules migrate to the site of HIV-1 assembly stabilized by both the actin and tubulin cytoskeleton (Fig. 2H) (Jolly *et al.*, 2007). The Gag precursor protein Pr55 plays a central role in virus assembly and is sufficient for production of non-infectious virus particles in the absence of other viral proteins (Gheysen *et al.*, 1989; reviewed by Resh, 2005). Unlike other enveloped viruses, HIV leaves the cell *via* one of two budding pathways depending on the cell type from which it is exiting. HIV released from T lymphocytes predominantly buds through the cell membrane and acquires its envelope components from the plasma membrane, whereas virus replicating in macrophages usually exits the cell *via* vesicles of the endosomal network (reviewed by: Kolegraff *et al.*, 2006; Bukrinskaya, 2007).

In T cells, HIV-1 is believed to assemble at and bud from so-called lipid rafts (Nguyen & Hildreth, 2000), plasma membrane (PM) microdomains that are enriched in cholesterol and sphingolipids and serve as platforms for protein–lipid interactions and for the assembly and/or budding of a range of enveloped viruses (reviewed by Waheed & Freed, 2009). The MA domain of Gag is responsible for its targeting and association with PM (Saad *et al.*, 2006). Membrane binding is mediated by insertion of the myristoyl group of MA into the lipid bilayer and by a patch of basic residues which binds acidic phospholipids, particularly phosphatidylinositol 4,5-bisphosphate (PIP_2). The interaction with lipid rafts promotes Gag oligomerization (Ono *et al.*, 2007) which, however, does not directly involve the MA domain. Instead, critical contacts are made by the C-terminal domain of CA, the adjacent SP1 spacer, the NC region, and, to some extent, the N-terminal domain of CA (reviewed by Ganser-Pornillos *et al.*, 2008). During virion assembly, the MA domain of Gag associated with the PM also facilitates envelope protein binding to the site of budding *via* an interaction with the transmembrane (gp41) subunit of Env. Mutations in MA that abrogate viral envelope incorporation into virions also prevent Env association with PM (Bhattacharya *et al.*, 2006; Bhatia *et al.*, 2009). The Gag-pol precursor, Pr160, cannot form virus particles on its own and is directed into the assembling particle also through its interaction with Gag (Smith *et al.*, 1993; Srinivasakumar *et al.*, 1995). The C-terminal fragment of the Gag domain in the Gag-pol polyprotein is proposed to be responsible for this interaction. Particularly, deletions in the region immediately C-terminal to the major homology region (MHR) of the HIV-1 CA in Gag-pol markedly affect both virus assembly and the incorporation of Gag-pol (Chien *et al.*, 2006).

Plasma membrane is the primary site of productive HIV-1 assembly in T lymphocytes as well as in most transformed cell lines (Finzi *et al.*, 2007b). Nonetheless, it has recently been shown that under some circumstances efficient virus production can occur intracellularly (Joshi *et al.*, 2009). In the case of macrophages, HIV-1 buds predominantly into endosomes or structures resembling multivesicular bodies (MVB) and is presumed to be released from these cells *via* exocytosis (Nguyen *et al.*, 2003; Kramer *et al.*, 2005). Since there is also a biological pathway that is used by a number of cell types, for example hematopoietic cells, to secrete microvesicles into the extracellular space, HIV might "hijack" that physiological pathway for leaving the infected cell (Gould *et al.*, 2003). Although the exact nature of the signals that direct proteins necessary for virus assembly to either cell membrane or exosome is not known, it is clear that two distinct pathways for HIV-1 Gag targeting exist, one to the lipid rafts of the PM and the other to the MVB (Ono & Freed, 2004).

Lentiviruses, as many other enveloped viruses, complete their replication cycle by budding through the cellular membrane (Fig. 2I). Since the virion envelope is derived from the host cell membranes, virus egress presents a problem because dissociation from the host membrane is not an easy or spontaneous step. In contrast to virus cell entry, when gp41 conformational changes provide free energy sufficient to promote membranes fusion (Fig. 2B) (see

section on Entry), no such viral protein is known to exist to overcome the energy barrier required for the membranes' separation. Thus, viruses have evolved mechanisms that usurp the physiological pathway used by cells to create cytoplasm-containing vesicles within endosomes. The observation that mutations in the p6 domain of Gag prevent the release of budded virus particles from the cell surface has shed light on this issue (Göttlinger *et al.,* 1991). Analysis of the p6 region revealed a PTAP amino acid motif, mutation of which was responsible for this defect (Göttlinger *et al.,* 1991; Huang *et al.,* 1995; Demirov *et al.,* 2002). This sequence interacts with cellular components of the endosomal sorting complex required for transport (ESCRT) pathway to mediate virus release (reviewed by: Bieniasz, 2006; Chen & Lamb, 2008; Carlton & Martin-Serrano, 2009).

An alternative and very efficient way for virus spreading is cell-to-cell transmission (Sato *et al.,* 1992). This transmission takes place at intercellular contact sites, so-called virological synapses (VS) that resemble immunological synapses. In the case of HIV-1, the VS is created by the recruitment of CD4 receptor, CXCR4 and CCR5 coreceptors, and lymphocyte function-associated antigen 1 (LFA-1) from the uninfected (target) cell and viral Env, Gag and adhesion molecules (intercellular adhesion molecule, ICAM-1) in polarized, lipid raft-like patches on the effector (donor) cell (Jolly *et al.,* 2004; 2007; Jolly & Sattentau, 2005; Wang *et al.,* 2009b). However, it has been demonstrated recently that HIV transmission between T cells occurs efficiently in the absence of adhesion molecules (lack of LFA-1–ICAM-1 interactions) and that the Env — CD4 interactions are the main driving force of the VS creation between infected and uninfected CD4+ T cells (Puigdomènech *et al.,* 2008). Interestingly, apart from the VS formation, HIV can be transmitted between T lymphocytes over relatively long distances using nanotubular connections, typical of these cells (Sowinski *et al.,* 2008).

Initially, virus particles are released from the infected cell in an immature form containing a spherical shell of structural proteins underneath the virus membrane (Gag–Gag lattice with interactions mediated primarily by the CA and SP1 domains), but not a central cone-like core (Fig. 2J). During or shortly after virus budding from the plasma membrane, viral protease cleaves the Gag and Gag-pol polyprotein precursors to generate the mature Gag and Pol proteins. This processing takes place as an ordered cascade of cleavage reactions (the last one liberates SP1 from CA), leading to the formation of the inner core (the NC/RNA complex condenses at the center), development of a conical-shaped core shell (CA lattice), and conversion of the immature virus particle into an infectious virion (Fig. 2A) (reviewed by: Bukrinskaya, 2007; Ganser-Pornillos *et al.,* 2008).

The mature, infectious virus is now able to initiate another round of infection in a new target cell.

CONSTRUCTION OF SAFE AND EFFICIENT HIV-1-BASED LENTIVIRAL VECTORS

Because of their unique ability to transduce nondividing cells, Lentivirus-based vectors have great potential for delivering therapeutic genes to cells. However, because of safety concerns due to the potential for horizontal and cross-species transmission of recombined chimeric lentiviruses, the design of such vectors should include fundamental safety principles.

To address such safety issues several monitoring assays have been developed to test for the presence of replication competent lentiviruses (RCLs) in vector preparations, the possibility of vector mobilization from transduced cells, the persistence of vector-positive cells, and the abnormal clonal expansion of vector-modified cells (discussed by: Connolly, 2002; Manilla *et al.,* 2005; Sastry & Cornetta, 2009). Useful tips for vector construction and methods to test the quality and safety of vector lots can be found in *Guideline on Development and Manufacture of Lentiviral Vectors* published on-line in 2005 by EMEA's Committee for Medical Products for Human Use (CHMP) (see List of websites [9]). Some general criteria to be considered when conducting risk assessments for research involving lentiviral vectors are discussed in a similar document, *Biosafety Considerations for Research with Lentiviral Vectors*, published in 2006 by the U.S. National Institutes of Health (NIH)'s Recombinant DNA Advisory Committee (RAC) (see List of websites [10]).

Generally, to minimize risk associated with manufacturing and use of lentiviral vectors, all nonessential genes coding for accessory proteins and responsible for virulence, should be removed from the vector sequence. Additionally, the vector genome is usually split into several parts with limited sequence overlap in order to reduce to a minimum the possibility of recombination, vector mobilization and the generation of RCLs.

EVOLUTION OF THE VECTOR DESIGN CONCEPT

The first systems using replication-defective HIV-1 vectors were described in the early 1990s. Helseth *et al.* (1990) presented a trans-complementation assay for measuring the replicative potential of HIV-1 envelope glycoprotein mutants. The method utilized two plasmids: one containing an HIV-1 provirus with a deletion in the *env* gene and a chloramphenicol acetyltransferase (CAT) gene replacing the

nef gene, and the *rev* and *env* either mutated or left intact under the control of the HIV-1 LTR. The idea to design a single-step replication assays with transient complementation of Env function was implemented to exclude the influence of proviral *cis*-acting mutations and multiple rounds of replication on the level of reporter gene expression.

In other initial experiments employing a Lentivirus as a gene delivery tool, HIV-1 *env* sequences were deleted and replaced by *Escherichia coli gpt* drug resistance gene driven by internal SV40 promoter (Page *et al.,* 1990; Landau *et al.,* 1991). The second plasmid provided either the gp120 glycoprotein or a heterologous Env protein. Although in this system the complete set of viral ORFs coding for regulatory and accessory proteins was present, HIV HXB2 molecular clone used for the plasmid construction was bearing attenuating mutations in *vpr*, *vpu*, and *nef*. Surprisingly, in the absence of Env, infected cells were able to produce non-infectious virions with the characteristics of gp120-containing particles, suggesting that Env is not necessary for virus particle formation. However, incorporation of envelope glycoproteins originating from different viruses allowed pseudotyped vectors to broaden their cell tropism.

In another early study the packaging component was separated into two different plasmids, one expressing the *gag*, *pol*, *tat*, and *vif* genes, and the other expressing *env* and *rev* (Poznansky *et al.,* 1991). This system provided *trans*-acting viral functions that permitted the transfer of the HIV-1-derived vector without the generation of RCL. The two-plasmid packaging system was later extensively modified in experiments conducted to verify the roles of HIV-1 *cis*-acting elements in gene transfer into cells (Parolin *et al.,* 1994). A plasmid expressing *gag*, *pol*, *vif*, and *tat* genes was constructed by replacement of the 5′ HIV-1 LTR with the cytomegalovirus (CMV) immediate-early promoter (CMVie) and deletion of the ψ sequence. The second plasmid expressed the *rev* and *env* genes under the control of HIV-1 LTR, but both plasmids contained heterologous polyA signals derived from SV40. The vector plasmids encoding a selectable marker neomycin phosphotransferase

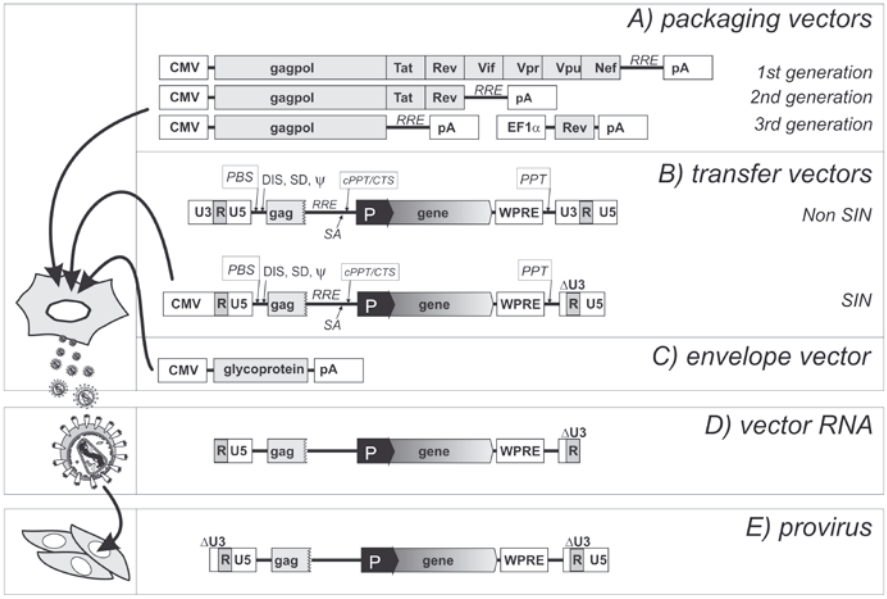

Figure 7. Lentivirus-based gene delivery system.
A–C. Plasmids used for transient transfection of producer cells. **A**. Packaging vector plasmids. First-generation vector contains all regulatory and accessory viral genes expressed from CMV promoter. Second-generation vector encodes only Tat and Rev proteins. Third-generation packaging system consists of two plasmids: one encoding Gag and Gag-pol polyproteins, the second — Rev protein. **B**. Transfer vector plasmids. In non-SIN vectors viral RNA is expressed from intact 5′ LTR. SIN (self-inactivating) vector bears deletion in U3 region (ΔU3), which inactivates transcription of entire viral RNA after provirus integration. **C**. Envelope vector. Depending on glycoprotein used, different viral pseudotypes are formed. **D**. Schematic representation of vector genomic RNA. In presented setting, cotransfection of either second-generation packaging vector or third-generation vectors along with SIN transfer vector and envelope vector results in formation of viral particles that contain dimeric RNA bearing ΔU3 mutation. **E**. After provirus integration into host cell DNA, transcription from mutated 5' LTR (duplicated 3' ΔU3) is abrogated.
Abbreviations: CMV, cytomegalovirus immediate-early promoter; EF1α, human elongation factor 1-α promoter; gag, 5' portion of *gag* gene containing dimerization/packaging signals; PBS, primer binding site; DIS, dimerization signal; SD, splice donor site; SA, splice acceptor site; ψ, packaging signal; cPPT, central polypurine tract; CTS, central termination sequence; RRE, Rev response element; PPT, polypurine tract; pA, polyadenylation signal; ΔU3, SIN deletion in U3 region of 3' LTR; P, internal promoter for transgene expression; WPRE, woodchuck hepatitis virus (WHV) post-transcriptional regulatory element.

(*neo*), contained the 5′ LTRs, PBS, ψ and 5′ SD, as well as 3′ genomic sequences, including PPT and LTR. Studies using these vectors revealed that the 5′ portion of the *gag* sequence inserted into the vector plasmid significantly enhanced marker gene transfer efficiency, possibly because a part of the HIV-1 packaging/dimerization signal is present in the extreme 5′ part of the Gag coding region (i.e., SL4; see section on Assembly and Budding and Fig. 6) (Clever *et al.,* 1995). A positive effect was also observed upon inserting RRE sequences but only for those vectors which contained *gag* sequences (Parolin *et al.,* 1994).

Other early HIV-1 vector systems involved the vesicular stomatitis virus G glycoprotein (VSV-G) and displayed relatively high titers. Additionally, such particles were easy to concentrate by ultrafiltration or ultracentrifugation and were able to infect a range of targets, including nondividing cells, demonstrating their superiority over simple retrovirus-based systems (Reiser *et al.,* 1996; Akkina *et al.,* 1996).

The further development of lentiviral vector systems was based on the concept of separating the *cis*-acting sequences that are essential for vector RNA synthesis, packaging, reverse transcription and cDNA integration, from the *trans* elements that encode viral enzymes as well as structural and accessory proteins. Hence, such a system typically consists of: a packaging expression cassette(s) (helper), a vector cassette (transfer vector), and an envelope expression cassette (Fig. 7A–C). This concept was originally developed in the context of gamma-retroviral vectors.

Packaging expression cassette(s) (helper plasmid)

The packaging cassette expresses viral enzymes and structural proteins necessary for infectious particle formation, with the exception of Env. The original first-generation lentiviral vector system developed in 1996 contained the accessory proteins Vpu, Vpr, Vif, Nef, and the Rev, and Tat regulatory proteins as functional components of the packaging plasmid. Further refinements included eliminating the LTRs as well as ψ and PBS sequences (Naldini *et al.,* 1996a; 1996b). This prevented the packaging of full-length mRNA encoding *trans* elements into nascent vector particles. However, RRE and the 5′ SD site remained unchanged, allowing normal mRNA processing and Rev-dependent export from the nucleus. In the absence of native LTRs, RNA synthesis was driven by promoters derived from other viruses, usually CMV or RSV (Rous sarcoma virus), whereas the polyA signal was adopted from SV40 or insulin gene (Fig. 7A).

These packaging constructs were subsequently refined by eliminating all accessory proteins that are associated with virulence and cytotoxicity (Table 1) and are not required for virus replication *in vitro* (Gibbs *et al.,* 1994), eventually leading to the creation of the second-generation packaging cassette (Zufferey *et al.,* 1997; Kafri *et al.,* 1997; Kim *et al.,* 1998; Mochizuki *et al.,* 1998). In this system only Rev and Tat proteins were expressed together with the Gag and Gag-pol polyproteins (Fig. 7A).

In order to further improve the biosafety of the system, *rev* was later placed on a second plasmid, while *tat* was completely removed. The Tat function was replaced using modified 5′ LTR enhancer/promoter elements containing strong, constitutive RSV- or CMV-derived promoters in the corresponding vector constructs (Fig. 7A). This systems is referred to as a third-generation packaging cassette (Dull *et al.,* 1998). In some ways it resembles the system described earlier by Parolin *et al.* (1994), but is much more streamlined. Fortunately, all those manipulations did not substantially affect vector production (titer) or infectivity. Moreover, the number of recombination events needed for the potential generation of an RCL increased considerably, and potential RCLs would not contain any proteins involved in wild-type virus virulence and pathogenicity.

However, homologous recombination was still possible since there were overlapping HIV sequences present in the helper and vector plasmids. These sequences encompass the RRE *cis*-element and a portion of the *gag* gene. RRE cannot be easily eliminated because it is necessary for efficient expression of HIV-1 *gag* and *pol* genes from the packaging construct. The reason for this requirement is low GC content and suboptimal codon usage in wild-type HIV mRNA. This causes instability of the RNA, which can be rescued from degradation by Rev binding. Codon optimization of the packaging cassette by changing the codon bias of the HIV-1 *gag-pol* to that of the human genome removed the homology with *gag* portion present in the transfer vector and led to efficient protein synthesis in the absence of Rev. This enhancement was shown to be mediated by increased mRNA stability and transport (Ngumbela *et al.,* 2008). Thus, it allowed construction of packaging cassettes that do not contain an RRE (Kotsopoulou *et al.,* 2000). It was also shown that production of the Gag-pol polyprotein from the codon-optimized mRNA is Rev-independent and that this RNA does not use the CRM-1-mediated nuclear export pathway. However, proper vector cassette expression remains Rev-dependent due to the requirement of a portion of *gag* for efficient packaging of the vector RNA (Parolin *et al.,* 1994). A complete removal of Rev from the system, even if the helper plasmid lacked the RRE sequence, resulted in a significant (five-fold) reduction of vector titers when compared with the Rev/RRE containing systems (Kotsopoulou *et al.,* 2000).

Other successful attempt to establish a Rev-independent packaging system was made by inserting the constitutive transport element (CTE) from Mason-Pfizer monkey virus (MPMV) into the HIV-1 sequence (Bray *et al.,* 1994). Rev co-expression was found to be dispensable for transgene expression from CTE-containing vectors and titers obtained were roughly equivalent to the titers obtained with Rev-containing vectors (Srinivasakumar *et al.,* 1997). The CTE function is similar to that of RRE (Rizvi *et al.,* 1996). Moreover, if it stabilizes RNA to a grater extent than RRE does or enhances polysome loading of unspliced RNA (Swartz *et al.,* 2007) it would be an additional benefit to put it in place of RRE. However, conflicting results with regard to the contribution of CTE itself have been reported (Kim *et al.,* 1998). Nevertheless, as demonstrated more recently by Oh *et al.* (2007), an individual copy of the CTE is unable to replace the RRE in enhancing the production of functional vector particles and the incorporation of the multiple CTE sequences, in both the transfer or packaging plasmid, is required to eliminate the need for the expression of the *rev* gene during vector production.

Safety improvement in the design of vector packaging systems is still an area of intensive research. For example, the sequences encoding HIV enzymatic and structural proteins were split into two cassettes: one expressing the Gag/PR polyprotein and the other — the Vpr/RT/IN fusion protein. This prevented vector-mediated transfer of a functional *gag-pol* sequence into target cells (Wu *et al.,* 2000; Kappes *et al.,* 2003).

Attempts to create hybrid lentiviral vector systems exploiting the limited sequence homology between other members of the genus have also been pursued. It was shown that HIV-1 vectors can be effectively packaged by SIV core particles (White *et al.,* 1999; Pandya *et al.,* 2001). HIV-2 transfer vector RNAs can be encapsidated by the HIV-1 packaging machinery, or alternatively, HIV-1 vector can be packaged by a chimeric construct with the leader-*gag* region of HIV-2 replaced with that of an HIV-1 packaging construct (Sachdeva *et al.,* 2007).

Furthermore, lentiviral particles can be produced using transient expression of lentiviral packaging genes from a recombinant adenoviral vector. In this experimental setting cells stably transfected with a SIV-based vector cassette were transformed into lentiviral vector producer cells by infection with adenoviral "lenti-pack" vectors (Kuate *et al.,* 2004). In a more recent study Semliki Forest virus (SFV) was used to produce recombinant pseudotyped HIV-1 virions. In the SFV/HIV hybrid system SFV-derived packaging cassettes allowed encapsidation of SFV/HIV-1 vectors bearing both *cis* and *trans* HIV elements, and enabled transfected cells to produce transducing-competent chimeric SFV/HIV particles (Del Vecchio *et al.,* 2009).

Vector cassette (transfer vector)

The vector cassette expresses full-length vector mRNA which contains all *cis*-acting elements necessary for efficient packaging, reverse transcription, nuclear import and integration into the host genomic DNA. Typically, the vector plasmid contains a transgene expression cassette with the gene of interest driven by an internal promoter, usually positioned between the 3′ Tat/Rev SA site and the 3′ LTR (Fig. 7B). The design of vector cassette has evolved in parallel with the development of packaging cassette. Early constructs contained intact 5′ and 3′ LTRs, and, thus, transcription was Tat-dependent. The resulting full-length transcript was comprised of TAR, PBS, SD and ψ sequences present in the 5′ UTR, a 300–400 bp 5′ portion derived from the *gag* gene (sometimes cloned out of frame or bearing nonsense mutation), RRE sequences, a splice acceptor (SA) site as well as PPT and polyA sequences within the 3′ LTR. The transgene with its own promoter was placed in between these sequences (Naldini *et al.,* 1996a; 1996b; Poeschla *et al.,* 1996).

Earlier reports showed that heterologous enhancer/promoters inserted into the HIV-1 LTR in place of the T cell-specific transcription factor (LEF-1/TCF-1α), NFAT, NF-κB, and Sp1 transcription factor binding sites in the U3 region (Fig. 5) drive transcription from the chimeric LTRs in the absence of Tat and that such modified viruses are replication-competent (Chang *et al.,* 1993). Weak transactivation by Tat was still evident and the infectivity of the viruses was cell type-dependent. Based on these early studies, Tat-independent transcription of the vector cassette was accomplished by replacement of the enhancer/promoter sequences in the U3 region of 5′ LTR with a strong heterologous promoter derived from CMV (Fig. 7B) (Kim *et al.,* 1998), as it had been done previously for the MLV (Soneoka *et al.,* 1995). Such transfer vectors are now an integral part of the third-generation packaging systems lacking Tat. However, transcription from such chimeric LTRs in producer cells still could be upregulated by Tat, which resulted in higher titers and increased transduction efficiency of newly made vector particles by 40% (Dull *et al.,* 1998). This could be explained by the fact that the TAR element is still present in nascent transcripts (Fig. 6).

Deletion of the enhancer/promoter sequences in the U3 region of 3′ LTR (120–400 bp; ΔU3) has led to the creation of so-called self-inactivating (SIN) vectors (Fig. 7B) (Dull *et al.,* 1998; Miyoshi *et al.,* 1998; Zufferey *et al.,* 1998; Iwakuma *et al.,* 1999). This idea was first introduced more than a decade earlier

in the context of MLV-derived retroviral vectors (Yu *et al.,* 1986). When viruses derived from such transfer constructs are used to infect cells, the deletion is reproduced in the 5′ LTR during reverse transcription, resulting in the transcriptional inactivation of the provirus (Fig. 7D, E). The most important improvement of viral vector safety offered by the SIN design is the reduction in expression of full-length transcripts available for packaging, which minimizes the possibility of vector mobilization upon superinfection with wild-type HIV (Bukovsky *et al.,* 1999). The chance of RCL formation is also reduced due to further elimination of sequences homologous to wild-type virus. Additionally, SIN deletion should make insertional mutagenesis less likely, preventing the transcriptional interference with promoter/enhancer elements present in the host genome. Actually, in the recently developed cell culture assay for insertional mutagenesis, SIN deletion of the promoter/enhancer region from HIV-based vector LTRs resulted in a complete abrogation of the insertional gene activation, frequently observed for both lentiviral and gamma-retroviral vectors (Bokhoven *et al.,* 2009).

Indeed, recent studies have identified transcriptionally active LTR as the major determinant of vector genotoxicity (Montini *et al.,* 2009). In a mouse tumor-prone HSPCs transplantation model, a lentiviral vector with a chimeric onco-retroviral LTR increased tumor onset to a level expected for a simple retrovirus-based vectors. In a parallel experiment, both lentiviral and gamma-retroviral vectors with a SIN LTR appeared to be neutral. It was concluded that in addition to the aforementioned transcriptional interference mechanism, the placement of enhancer/promoter sequences in the LTR upstream of a strong splice donor site may increase the probability of chimeric transcript formation and oncogene activation. Hence, for a safe vector design SD sites downstream of strong promoters should be avoided. In addition to the use of SIN-modified LTRs, moderately active internal promoters are recommended for vectors for clinical applications, especially when the weak 3′ LTR polyA signal serves to terminate transcription (see Heterologous PolyA Signals section below).

Integrated vector genomes containing full-length 5′ LTRs (Bukovsky *et al.,* 1999; Evans & Garcia, 2000; Levine *et al.,* 2006) can be mobilized by infection with wild-type HIV virus. It has been shown that virtually all integrated proviral genomes are transcribed, albeit at low frequency dependent on the integration position. On the other hand, in the experiments employing T cells, only a small number of integrated HIV-1-based vectors expressed transgene from viral LTR. The vast majority of the vectors was silenced immediately after integration (Mok *et al.,* 2007). Although SIN proviruses are much less likely to be mobilized, any transcript derived from a cryptic promoter present within the integrated sequences or in the surrounding host DNA, that includes the R region of the 5′ LTR, may give rise to an intact proviral DNA genome that can be reintegrated (Logan *et al.,* 2004a; Hanawa *et al.,* 2005). The mobilization of integrated SIN vectors during wild-type HIV superinfection has not been demonstrated yet, but careful design of a safe vector requires further efforts to resolve this potential problem (see section on Chromatin Insulators below).

Another possible source of viral genomes available for packaging are episomal forms of the vector DNA that circularizes and remains unintegrated in transduced cells. Such episomal forms of viral DNA, single- and double-LTR (1-LTR and 2-LTR) circles, are diluted upon cell division (Van Maele *et al.,* 2003), but in nonproliferating, quiescent cells they are stable and transcriptionally competent (Gillim-Ross *et al.,* 2005) and, thus, can considerably contribute to the availability of full-length gRNAs for packaging and mobilization. However, in more recent study on HIV-1 preintegration transcription, the majority of 2-LTR circles appeared not to be active in directing RNA synthesis. It was concluded that the nonintegrated templates were mainly from the predominant DNA species, such as the full-length, linear DNA (Iyer *et al.,* 2009).

Envelope expressing cassette

The last element of a lentiviral vector system is an envelope cassette, which provides nascent particles with envelope glycoprotein (Fig. 7C). Since the genuine lentiviral envelope gene is removed from the system, an additional plasmid expressing a heterologous glycoprotein is used during vector production. Such an approach, called pseudotyping, offers several significant advantages: 1) increase in vector safety due to elimination of sequence homology with wild-type virus, 2) expanded or selective specificity of pseudotyped vector tropism towards target cells, 3) improvement of particle stability allowing virus concentration and long-term virus stock storage.

As was described above, Page *et al.* (1990) and Landau *et al.* (1991) were the first to demonstrate the ability of a recombinant Lentivirus-based vector to incorporate a heterologous envelope glycoproteins. They achieved relatively high titers (10^5 colony forming units per ml, CFU/ml, of preparation) and an altered cell tropism of the HIV-1 based vectors using eco- and amphotropic MLV Env or HTLV-I Env. Nevertheless, the glycoprotein of choice for the vast majority of vector systems currently in use is VSV-G. It offers unequaled titers (10^7–10^8 transducing units per ml, TU/ml, before concentration), excellent stability of the virions and

allows virtually all mammalian (and insect) cell types to be infected. The first successful attempts to pseudotype lentiviral vectors with VSV-G were conducted concurrently by several groups (Naldini *et al.,* 1996a; Reiser *et al.,* 1996; Akkina *et al.,* 1996; Poeschla *et al.,* 1996). The VSV-G envelope facilitates vector entry *via* the endocytic pathway, which diminishes the requirement for viral accessory proteins for full infectivity (i.e., Nef; see section on Entry) (Aiken, 1997; Chazal *et al.,* 2001). However, the mechanism responsible for cell binding as well as cellular receptors for this glycoprotein remain unknown. For example, an involvement of phosphatidylserine, an ubiquitous lipid of the cell membrane that was long believed to serve as a receptor for VSV-G, is currently controversial (Coil & Miller, 2004; Carneiro *et al.,* 2006).

One drawback of the production of pseudotyped vectors using VSV-G is that, due to its fusogenic properties (Li *et al.,* 1993), it is toxic to some mammalian cells (e.g., 293 cells) if constitutively expressed (Burns *et al.,* 1993). Hence, the use of producer cell lines expressing packaging proteins for long-term virus production requires conditional production of this glycoprotein. Such retrovirus/Lentivirus producer cell lines have been constructed with tetracycline-regulatable promoters used to express VSV-G (Chen *et al.,* 1996; Ory *et al.,* 1996; Kafri *et al.,* 1999; Klages *et al.,* 2000; Farson *et al.,* 2001; Ni *et al.,* 2005; Cockrell *et al.,* 2006; Broussau *et al.,* 2008).

Another shortcoming for application of VSV-G-pseudotyped vectors *in vivo* is the complement- and antibody-mediated immune response directed against the envelope (DePolo *et al.,* 2000; Higashikawa & Chang, 2001). The complement-mediated inactivation can be alleviated by chemical modifications of the envelope glycoprotein. Successful extension of the vector half-life and an increase in transduction efficacy *in vivo* have been demonstrated with VSV-G conjugated with poly(ethylene glycol) (PEG) (Croyle *et al.,* 2004).

Targeting lentiviral vectors by pseudotyping

The construction of optimal or tissue-specific envelopes is still one of the most important research objectives. For example, a chimeric RD114 glycoprotein, with the transmembrane and extracellular domains of the feline endogenous virus RD114 glycoprotein fused to the cytoplasmic domain derived from the amphotropic MLV 4070A Env, enables transduction of bone-marrow-derived mesenchymal stem cells (MSCs) with an efficiency

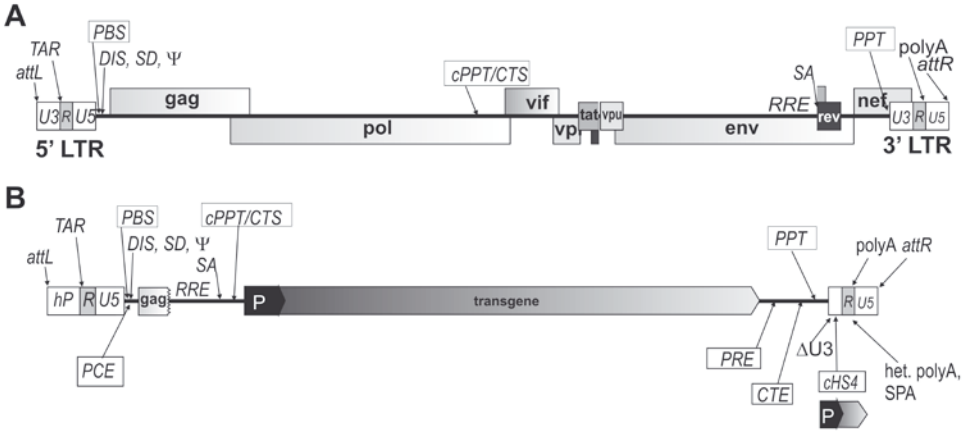

Figure 8. Comparison of wild-type HIV-1 provirus and optimized lentiviral vector.
A. Schematic representation of HIV-1 provirus. **B**. Typical *cis*-acting elements are marked above the vector. Below — examples of additional modifications introduced to Lentivirus-based vectors in order to elevate titer and/or transgene expression levels. After transfection, viral genomic RNA is synthesized using strong hybrid promoter placed at 5′ end of vector. Elimination of weak promoter/enhancer from 5′ LTR enables Tat-independent transcription and use of third-generation packaging system. Incorporation of post-transcriptional control element improves translation efficiency. In SIN (self-inactivating) vectors deletion in U3 region of 3′ LTR (ΔU3) is duplicated during reverse transcription and integration, which results in abrogation of viral RNA transcription. ΔU3 site can be used for insertion of chromatin insulator or transgene cassette and, thus, these elements will be duplicated in provirus. To improve polyadenylation of SIN vector transcript, heterologous or synthetic polyadenylation signals can be inserted into R region of 3' LTR. Post-transcriptional regulatory elements and RNA transport elements can be incorporated into 3' end of transgene to reinforce transcript 3' end formation, RNA stability and nuclear export.
Abbreviations: hP, hybrid promoter; *att*L, *att*R, left and right attachment sites; TAR, transactivation response element; gag, 5′ portion of *gag* gene containing dimerization/packaging signals; PBS, primer binding site; DIS, dimerization signal; SD, splice donor site; SA, splice acceptor site; ψ, packaging signal; cPPT, central polypurine tract; CTS, central termination sequence; RRE, Rev response element; PPT, polypurine tract; polyA, polyadenylation signal; ΔU3, SIN deletion in U3 region of 3' LTR; P, internal promoter for transgene expression; PCE, post-transcriptional control element; PRE - post-transcriptional regulatory element; CTE, constitutive transport element; cHS4, chicken β-globin insulator; het. polyA, heterologous polyA signal; SPA, synthetic polyA signal.

similar to that obtained with VSV-G pseudotypes, but with lower cytotoxicity (Zhang *et al.,* 2004a).

In another study, lentiviral vectors pseudotyped with a glycoprotein derived from the Rabies virus PV strain exhibited the best performance and neuronal tropism among the tested envelopes. Rabies PV virus-pseudotyped vectors transduced neurons and other cells of neuronal origin with unequaled efficiencies when compared to other Lyssavirus glycoproteins, including VSV-G (Federici *et al.,* 2009). Furthermore, the Rabies envelope glycoprotein facilitates *in vivo* retrograde transport of vector particles along neuronal axons, enabling remote gene delivery to the central nervous system (CNS) *via* peripheral virus injection (Mazarakis *et al.,* 2001; Federici *et al.,* 2009).

A serious disadvantage of the lentiviral vectors pseudotyped with VSV-G is their inability to infect cells blocked in the G0 phase of the cell cycle. However, a high level transduction of such relevant therapeutic targets as memory and naïve T cells was achieved by the incorporation into virions glycoproteins from Edmonston measles virus (MV). Presence of hemagglutinin, responsible for receptor recognition, and the fusion protein on the virus surface allowed the efficient transduction of resting cells *via* the MV receptors: SLAM and CD46 (Frecha *et al.,* 2008a). Similarly, lentiviruses pseudotyped with the MV glycoproteins were able to infect quiescent B lymphocytes as well as leukemic cancer cells, B-CLL cells, blocked in the G0/G1 early phase of the cell cycle (Frecha *et al.,* 2009). Another research group reported transduction of unstimulated primary B cells using retargetable MV envelope glycoproteins (Funke *et al.,* 2008). In this study, hemagglutinin was engineered to display either the epidermal growth factor (EGF) or a single-chain antibody directed against CD20. Thus, cell infection was performed not through the MV receptor but *via* the EGF and CD20 receptors. Consequently, gene transfer to cells that expressed the targeted receptor was several orders of magnitude more efficient than to other cells.

In another recent report, a targeted lentiviral vector exploiting a natural ligand–receptor binding mechanism was used for modification of c-KIT (CD117) receptor-expressing cells *in vitro* and *in vivo*. To target CD117+ cells the vector surface was engineered to contain membrane-bound human stem cell factor (hSCF) (for specific receptor recognition) and a Sindbis virus glycoprotein-derived fusogenic molecule (FM) (for membranes fusion) (Froelich *et al.,* 2009). The targeted vector was 3.6–5 times more effective in transducing CD117+ cells than control viruses. Similar strategy was used to target CD20-expressing B cells (Yang *et al.,* 2006), cells expressing monospecific surface immunoglobulin recognizing CD20 (αCD20) (Ziegler *et al.,* 2008), dendritic cells expressing dendritic cell-surface protein DC-SIGN (Yang *et al.,* 2008b), and CD3+ T cells (Yang *et al.,* 2009). Notably, engineering (mutagenesis) of the fusion-active domain of the FM enabled enhancement of targeted transduction by a factor of 8–17, possibly due to a wider pH range of activity observed in engineered FMs (Lei *et al.,* 2009).

The strategies used for Lentivirus-based vector pseudotyping have been comprehensibly reviewed by: Verhoeyen & Cosset (2004); Cronin *et al.* (2005); and Frecha *et al.* (2008b).

ADDITIONAL ELEMENTS FOR VECTOR IMPROVEMENT

Since HIV-1 virus can effectively package approx. 9.7 kb of its genome, reduction of the length of the viral vector backbone should increase its payload carrying capacity. In the work published by Cui *et al.* (1999), subsequent mutations and deletions of the viral regulatory elements, including SD, most of *gag*, and RRE, have brought down the content of the original HIV-1 sequences in the vector DNA to less than 550 bp. This should allow accommodation of more than 9 kb of foreign DNA. Yet the usefulness of such design was diminished by a decrease in vector titer, about half that of the wild-type construct. In another detailed study on lentiviral vector capacity, maximal vector size (including 1.6–2.2 kb of the virus backbone) has been estimated to approx. 13.5 kb (Kumar *et al.,* 2001).

Thus, the design of a safe and efficient lentiviral vector requires both deletions of non-necessary sequences from the backbone and insertions of elements that are proven to have a positive effect on vector titer or transgene expression (Fig. 8).

cPPT–CTS

The role of cPPT and CTS in the HIV-1 life cycle was described in previous sections. Consistently with the *cis*-acting role of the central DNA Flap (about 170 bp) in reverse transcription and in the nuclear import of viral PIC, its re-insertion into HIV-1-derived gene transfer vectors strongly stimulates gene transfer efficiencies (up to ten-fold) in all cell types tested, both *in vivo* and *ex vivo* (Sirven *et al.,* 2000; Park & Kay, 2001; Zennou *et al.,* 2001; Dardalhon *et al.,* 2001; Baekelandt *et al.,* 2002; Manganini *et al.,* 2002; Nguyen *et al.,* 2002; Van Maele *et al.,* 2003; Breckpot *et al.,* 2003; Giannini *et al.,* 2003; Ao *et al.,* 2004; Logan *et al.,* 2004b). Interestingly, this positive effect of cPPT and CTS on the transduction efficiency depends neither on the position and number of DNA Flaps in the vector backbone nor on the Flap sequence (De Rijck *et al.,* 2005; De Rijck & Debyser, 2006).

PRE and PCE

Another commonly used *cis*-acting element is the woodchuck hepatitis virus (WHV) post-transcriptional regulatory element (PRE), WPRE (Zufferey *et al.,* 1999). Incorporation of this sequence (600 bp) in the 3′ untranslated region of a transcript increases overall transgene expression by more than six-fold (Oh *et al.,* 2007). In the context of the FIV vector, a WPRE-containing construct gave a functional titer about ten times higher than the WPRE-lacking one (Pistello *et al.,* 2007). Interestingly, in this experimental setup insertion of cPPT had no noticeable effect on the vector performance.

One should note that the WPRE element contains an ORF encoding a truncated peptide of the WHV X protein (WHX), which might be associated with hepatocellular carcinomas. However, neither expression of this peptide from a lentiviral vector nor its direct involvement in oncogenesis have been demonstrated. Besides, the potentially harmful sequences can be successfully eliminated from WPRE without negative effects on its functionality, which enables designing safe and efficient vectors for *in vivo* applications (Schambach *et al.,* 2006; Zanta-Boussif *et al.,* 2009).

The exact role of WPRE in vector performance is unclear. One of its functions may be the reduction of viral readthrough transcription and, thus, improvement of transcript termination leading to higher titers and transgene expression (Higashimoto *et al.,* 2007). Indeed, in a situation when the SIN design negatively affected promoter performance (see section Heterologous PolyA Signals below) insertion of WPRE near the 3′ LTR reversed this effect (Salmon *et al.,* 2000a). Post-transcriptional regulatory elements may additionally enhance intronless transgene expression through stabilization of the 3′ end of the transcript and improvement of the nucleocytoplasmic export of unspliced mRNAs. Also other than WPRE *cis*-acting elements (e.g., hepatitis B virus (HBV) PRE, HPRE) can be considered useful in *in vivo* applications (Sun *et al.,* 2009).

Another *cis*-acting post-transcriptional element that may positively affect transgene expression is PCE (see section on Gene Expression). When the CMVie enhancer and spleen necrosis virus (SNV) PCE were inserted into a lentiviral vector backbone, they acted together to achieve coordinate increases in RNA synthesis and translation (Yilmaz *et al.,* 2006). It was demonstrated that CMVie and PCE function synergistically to increase protein yield in transduced cells four-fold compared to a control vector lacking these sequences.

Heterologous polyA signals

Due to deletions in the U3 region containing polyadenylation "enhancers" (DeZazzo *et al.,* 1992), SIN vectors suffer from leaky transcription termination increasing the probability of readthrough into cellular genes, potentially oncogenes. Hence, incorporation of strong polyA signals is of great importance for safe vector design. An attempt to solve this problem was made by Schambach *et al.* (2007). Insertion of a 100 bp sequence, comprising a recombinant direct repeat of the upstream polyadenylation enhancer element (or upstream sequence element, USE) derived from SV40, improved both the titer and gene expression from a viral vector. Additionally, this element suppressed readthrough more efficiently than WPRE did and was able to substitute for the WPRE functions to some extent.

Furthermore, replacement of the original polyA signal in the R/U5 regions of 3′ LTR with a bovine growth hormone polyadenylation (bGHpA) sequence significantly elevated the efficiency of SIN vectors (Iwakuma *et al.,* 1999). The bGHpA signal was also successfully used to enhance expression of the second gene in vectors carrying multiple genes (Osti *et al.,* 2006). However, insertion of a polyA signal between two LTRs in a lentiviral vector very often leads to a premature termination of the genomic transcript and greatly decreases the viral titer. Thus, the aforementioned experiments were conducted transient, only in cells transfected with the transfer vector plasmid. Indeed, the internal polyadenylation signals in lentiviral vectors reduce the viral titers. This undesired effect is promoter-dependent and can be avoided by using selected promoters (Hager *et al.,* 2008). A similar positive effect on downstream gene expression was achieved when a synthetic *polyadenylation* (*SPA*) termination sequences were used and, to avoid disruption of viral mRNA production by the polyadenylation sequences in the middle of the vector, the expression cassettes were cloned in the antisense orientation relative to the viral LTRs (Tian & Andreadis, 2009).

Chromatin insulators

Previously, Logan *et al.* (2004a) and Hanawa *et al.* (2005) reported the potential of transcription of integrated SIN vectors from cryptic promoters, either within or upstream of the vector genome. Worse still, particles containing such mobilized vector genome were capable of transferring the intact proviral DNA into target cells. In order to minimize such an undesirable phenomenon, a 1.2 kb fragment of the insulator element from the locus control region of the chicken β-globin gene, chicken hypersensitive site 4 (cHS4) sequence (Chung *et al.,* 1993), was inserted into the vector LTRs. This resulted in a significant (to one-fourth of the control level) reduction in the full-length vector transcription (Hanawa *et al.,* 2005).

On the other hand, insertion of insulator sequences into vectors can help maintain long-term

transgene expression by suppression of chromosomal position effects (transgene silencing) resulting from integration into the host chromatin (Ramezani *et al.,* 2003; Hino *et al.,* 2004; Arumugam *et al.,* 2007). The insulator sequence was also successfully used to facilitate expression of the second gene in vectors carrying multiple genes (Osti *et al.,* 2006; Tian & Andreadis, 2009), to diminish clonal dominance in cultures of human T cells containing integrated vector genomes (Evans-Galea *et al.,* 2007), and to reduce basal expression from a regulatable promoter by shielding the vector from the effects of enhancer elements present in the neighboring host DNA (Pluta *et al.,* 2005; Vieyra & Goodell, 2007).

Fortunately for vector design, the size of the cHS4 insulator can be reduced to 400 bp without impairing its barrier function (Aker *et al.,* 2007), or even to 77 bp if combined with a homologous region from the human T cell receptor alpha/delta BEAD-1 insulator (Ramezani *et al.,* 2008). This is important, as lower viral titers were sometimes reported when doubled copies of full-length cHS4 were used to flank the expression cassette or when this large insulator fragment was cloned into 3′ LTR (Hanawa *et al.,* 2009; Nielsen *et al.,* 2009; Urbinati *et al.,* 2009). Observations suggest that this effect is caused by impaired RNA processing during transduction of the target cell, probably on the level of reverse transcription and integration. Hanawa *et al.* (2009) reported incorporation of the 250 bp-long core element of the cHS4 insulator. Use of this element rescued vector titer compared to the titer of 1.2 kb insulator-bearing virus. Additionally, the presence of this short core element significantly increased transgene expression from an internal promoter due to improved transcriptional termination and reduced the variability of expression caused by the position effects. However, results presented by other researchers showed that the core alone did not insulate viral vectors effectively and only combination of the core and distal 400 bp of cHS4 sequences restored full insulator activity (Arumugam *et al.,* 2009).

In summary, the evolution of the packaging cassette design and the optimization of the transfer vector have eventually led to the removal from lentiviral vector system of at least five proteins associated with HIV virulence, Tat, Nef, Vif, Vpr, and Vpu. This engineering coupled with pseudotyping makes the vectors based on lentiviral genome clearly distinct from the HIV virus in terms of their biology and, in consequence, ensures their safety as a gene delivery system. Furthermore, additional elements that are commonly incorporated into the vector backbones (e.g., cPPT and CTS, WPRE, PCE, heterologous polyA signals, and chromatin insulators) facilitate production of high titer virus stocks and elevate transgene expression levels.

NONINTEGRATING LENTIVIRAL VECTORS

The problem of a presence of the episomal, circularized forms of the vector DNA in infected cells was discussed previously in a context of the potential sources of viral genomes available for undesired mobilization and packaging (Vector Cassette (Transfer Vector) section). Nonetheless, recognition of the unique features of these LTR-containing circles has recently led to the development of so-called integration-deficient lentiviral vectors (IDLVs) or nonintegrating lentiviral vectors (NILVs) (for relevant review see: Sarkis *et al.,* 2008; Wanisch & Yáñez-Muñoz, 2009). In brief, the IDLVs resemble integrating vectors described in previous sections except that they have mutated integrase. Thanks to their episomal nature (integration frequencies are 3–4 logs below those of their integrating counterparts), the IDLVs have a greatly reduced risk of promoting insertional mutagenesis. Moreover, since active IN is required for the viral replication nonintegrating vectors should not support the HIV-1-based replication if an unlikely recombination event generated an RCL. In the vectors designated for use in gene therapy additional mutations at the *att* sites would reduce integration frequencies of the vector mobilized by a wild-type virus.

The HIV-1-based vectors packaged with a mutated integrase maintain transgene expression *in vitro* and *in vivo*. Because they are integration- and replication-deficient this expression is transient in dividing cells. In nondividing cells, however, gene transfer by the IDLVs is relatively stable (Philippe *et al.,* 2006). An episomal character of the nonintegrating vectors has also consequences in the stability of transcription since the IDLVs may not be a subject to the epigenetic silencing. Unfortunately, transgene expression levels observed for nonintegrating lentiviral vectors were much lower compared to those of their integrating counterparts (Bayer *et al.,* 2008). Another shortcoming of the IDLVs is their reduced transduction efficiency (Okada *et al.,* 2009).

Regardless of these limitations, nonintegrating lentiviral SIN vectors can be packaged in producer cells with an efficiency comparable to that of regular vector RNA (Ma & Kafri, 2004). Moreover, the Lentivirus-based episomal system for gene delivery proved its usefulness in the genetic correction of the disorder in animal models (Yáñez-Muñoz *et al.,* 2006), effective vaccination (see references in section on Lentiviral vectors in clinical gene therapy applications), gene silencing mediated by RNA interference (RNAi) (Wanisch & Yáñez-Muñoz, 2009), transgene delivery to the adult and fetal CNS (Rahim *et al.,* 2009) and to muscle *in vivo* (Apolonia *et al.,* 2007) as well as in the transposase-directed genomic integration of the minimal gene expression cassette (Vink *et al.,* 2009; Staunstrup *et al.,* 2009).

TARGETED INTEGRATION OF LENTIVIRAL VECTORS

The combination of episomal lentiviral vectors with the transposon-mediated transgene integration constitutes another step toward the construction of a safer gene delivery tool. The IDLVs provide an efficient gene delivery vehicle for the system whereas transposase directs transgene integration away from the transcriptionally active *loci*, favored by the regular integrase-proficient lentiviral vectors. This fact along with the absence of viral LTRs in a host cell genome minimizes the risk of the insertional mutagenesis or a proto-oncogene activation (Vink *et al.*, 2009; Staunstrup *et al.*, 2009).

Nonetheless, for gene therapy purposes, including either the gene repair or knock-down, the site-directed integration is highly desirable. In spite of the fact that the cells with modified genes can be isolated or enriched in the transduced/transfected *ex vivo* culture, the frequencies of derivation of such corrected cells are very poor with traditional gene targeting methods. An interesting attempt to achieve targeted gene repair was reported by Cornu & Cathomen (2007). The system, developed for the correction of the endogenous mutation in the marker gene directly in cellular genome, was based on the IDLVs-mediated delivery of the repair template and the I-*Sce*I yeast meganuclease-directed homologous recombination. Expression of the nuclease from separate vector created a double-strand break within the target *locus*, which enabled recombination with the template DNA. The marker expression was rescued in up to 12% of the cells. Although these results are very promising, described above method requires the initial engineering of recognition sites for the I-*Sce*I nuclease. Similar mechanism of the site-directed integration was also demonstrated with another non-viral enzyme, yeast Flp recombinase (Moldt *et al.*, 2008).

The possibility to avoid host genome manipulations is offered by the application of so-called designer nucleases that are able to cut target DNA at specific genomic sequences. Recent attention focuses on the chimeric zinc-finger nucleases (ZFNs) that can be engineered to target desired sites. Lombardo *et al.*

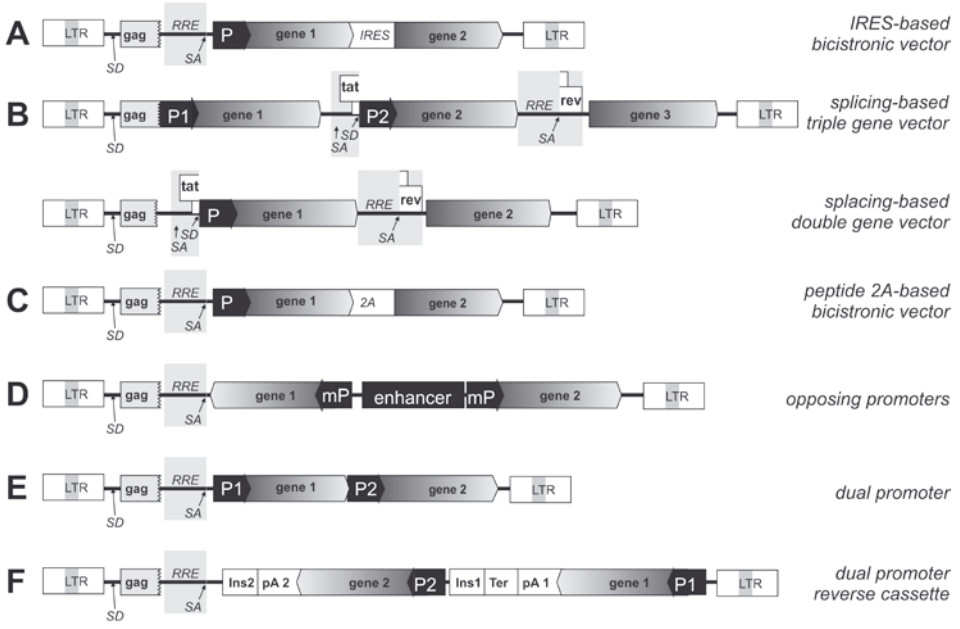

Figure 9. Transfer vectors for multigene expression.
A. Bicistronic vector based on IRES. Incorporation of IRES sequences from various viral genomes enables expression of more than one gene from single transcription unit. **B**. Multigene-expressing vectors based on splicing of mRNA. Multigene vector design includes utilization of one or two internal promoters (double- and triple gene cassette, respectively) and LTR-driven transcription of completely spliced mRNA (two or three independent transcriptional units). **C**. Bicistronic vector with peptide 2A sequence. Peptide 2A sequence enables fragmentation of nascent polyprotein through ribosomal skip mechanism, giving rise to two proteins encoded by single transcription unit. **D**. Expression of two genes from opposing promoters. Synthetic bidirectional promoter that consists of sequences of efficient promoter flanked by minimal promoter elements drives expression of divergent transgenes. **E**. Dual promoter-driven expression. Two transcriptional units are positioned in tandem forward orientation. **F**. Dual promoter-driven expression from reversed transcription cassettes. Two expression cassettes, inserted in reverse orientation with respect to LTRs, are separated by polyadenylation, terminator and insulator sequences.
Abbreviations: gag, 5′ portion of *gag* gene containing dimerization/packaging signals; SD, splice donor site; SA, splice acceptor site; RRE, Rev response element; IRES, internal ribosome entry sequence; 2A, peptide 2A sequence; pA, polyadenylation signal; Ins, chromatin insulator; Ter, transcription terminator; P1, P2, internal promoters; mP, minimal promoter.

(2007) used integrase-defective lentiviral vectors to express ZFNs and template DNA in order to obtain gene correction in different cell types. Successful target gene editing was achieved in up to 39% of the cells. The authors also demonstrated the site-specific gene addition in up to 50% of the cells in the cell lines, albeit human embryonic stem (ES) cells were much less susceptible for the IDLV/ZNF-mediated DNA integration (less than 5% of the cells).

METHODS FOR TRANSGENE EXPRESSION

Lentivirus-based vectors have emerged as convenient and versatile tools for gene delivery and long-term controllable expression of genetic information in target cells and tissues *in vitro* and *in vivo*. Many studies, including *in vivo* experiments and gene therapy trials, may benefit from simultaneous expression of two or more genes from one vector. For example, delivery of the therapeutic gene(s) can be monitored by the presence of an accompanying product of marker gene(s), easily detected using enzymatic reactions or by its fluorescence. In *ex vivo* experiments, the population of transduced cells can be enriched based on marker gene expression (e.g., by drug resistance or fluorescence activated cell sorting, FACS). Last but not least, expression of a marker gene from an additional ORF helps in evaluation of the vector titer.

Some experimental and clinical settings require conditional expression of the gene of interest, which is possible using regulatable promoters. The most popular methods for controlled transgene expression are based on the presence of a specific activator or repressor in the same cell. Hence, for efficient and reliable control, co-expression of these two elements from one vector would be highly advantageous.

For genetic correction of metabolic disorders, both in animal models and clinical applications, tissue-specific expression from a lentiviral vector is one of the most attractive possibilities. As was mentioned in the previous sections, pseudotyping of the vector with a tissue-specific envelope glycoprotein was shown, in a number of experiments, to be effective in delivering the transgene to the desired cells. Another valuable possibility of target-restricted expression is offered by the use of tissue-specific promoters that are exclusively or primarily active in an environment of the specific cell.

MULTIGENE VECTORS

IRES and splicing-based transgene expression

The idea of co-expression of more than one gene from a single lentiviral vector has been realized by different approaches. Reiser *et al.* (2000) described several sets of constructs, including bicistronic vectors and multigene vectors, expressing up to three exogenous genes from two or three different transcriptional units. Bicistronic expression cassettes (Fig. 9A) utilize a single heterologous promoter driving two separate ORFs linked by an IRES sequence. Expression of the second gene depends on the strength of the promoter (the ability to produce long transcripts efficiently, often in a cell-dependent manner) and on the performance of the IRES (in this experimental setup, Gtx IRES, derived from the 5′ UTR of the mRNA encoding the Gtx homeodomain protein, seems to be superior to the IRES derived from encephalomyocarditis virus, ECMV IRES; see also: Wang *et al.*, 2005). A multigene vector design (Fig. 9B) involves utilization of one or two internal promoters (double- and triple gene cassette, respectively) and LTR-driven transcription of completely spliced mRNA. Thus, in contrast to a bicistronic vector, two or three independent transcriptional units are formed. Due to the fact that the last gene in the construct is driven by the LTR (fully spliced mRNA), its expression is Tat (but not Rev)-dependent. However, the LTR-driven expression levels varied between transduced cell lines. It was possibly caused by differences in cell-type-specific splicing kinetics (Reiser *et al.*, 2000).

A similar vector design, employing IRES and natural HIV-1 splicing signals, was presented by Zhu *et al.* (2001b). Again, expression of the downstream gene was lower (by about 50%), both in ECMV IRES-containing and splicing-based vectors. Interestingly, in the absence of Rev, expression of the second gene from spliced mRNA was relatively higher, probably due to increased amounts of doubly spliced RNA in the cytoplasm.

Bi- and multicistronic vectors effective both *in vivo* and *in vitro* were developed also by other groups (Stripecke *et al.*, 2000; Mitta *et al.*, 2002; Richard *et al.*, 2004; Ben-Dor *et al.*, 2006; Sangiolo *et al.*, 2007; Lourenço *et al.*, 2009) using different IRES sequences, including those derived from EMCV, hepatitis C virus (HCV) or poliovirus. An IRES was also included in the helper packaging construct used for the first clinical study in anti-HIV therapy (Lu *et al.*, 2004; Levine *et al.*, 2006). However, published data evidence that initiation of translation from an IRES is less efficient than from the 5′-end of the cap-containing transcript (Mizuguchi *et al.*, 2000; Zhu *et al.*, 2001b; Yu *et al.*, 2003b; Osti *et al.*, 2006; Chinnasamy *et al.*, 2006; Khare *et al.*, 2008; Ibrahimi *et al.*, 2009).

Peptide 2A

To bypass the need for an IRES sequence, several groups used 2A peptide sequences (reviewed by Szymczak & Vignali, 2005) (Fig. 9C) to

construct bicistronic and polycistronic vectors effectively expressing multiple genes both *in vitro* and *in vivo* (Chinnasamy *et al.,* 2006; 2009; Yang *et al.,* 2008c; Hu *et al.,* 2009a; Ibrahimi *et al.,* 2009; Froelich *et al.,* 2009). The advantage of using 2A peptide in the construction of bicistronic vectors is its ability to co-express both genes at nearly equal levels. The 2A, or similar peptides, are used by many positive strand RNA viruses to "cleave" their polyproteins in order to produce mature proteins. The 2A peptide, encoded by a sequence separating the *2A* and *2B* genes, exhibits a cleavage-like activity possibly through a ribosomal skip during translation. When this sequence is incorporated between heterologous ORFs, this apparent cleavage leaves about 18 amino acids of the 2A peptide at the C-terminus of the first protein and a single proline at the N-terminus of the second one. Thus, a potential drawback of the use of 2A peptide is the possibility of inappropriate subcellular targeting of proteins (de Felipe & Ryan, 2004), therefore further manipulations, e.g., addition of a furin cleavage site, are sometimes recommended (Yang *et al.,* 2008c).

Bidirectional synthetic promoters

In search for efficient methods of multiple gene expression, Amendola *et al.* (2005) introduced synthetic bidirectional promoters to obtain divergent transcription of two mRNAs in the lentiviral vector. In the design of such synthetic bidirectional promoters a minimal core promoter is joined upstream to a strong promoter cloned in the opposite orientation (Fig. 9D). The rationale of this approach is that the upstream enhancer sequences in the efficient promoter, when flanked by minimal promoter elements, drive transcription in both directions. Lentiviral vectors containing such promoters, either constitutive or tissue-specific, enabled dual gene transfer into several tissues *in vivo*. Bidirectional vectors may be most useful for applications in which moderate transgene expression levels are desirable (Amendola *et al.,* 2005; Ibrahimi *et al.,* 2009).

The bidirectional concept system was later adapted for transcriptional amplification strategies (TASs) (Liu *et al.,* 2008). In this strategy, the 5′ cassette encoded an artificial transcriptional activator expressed from the minimal CMV promoter in reverse orientation. The downstream cassette allowed expression of the gene of interest from a tissue-specific promoter containing an upstream binding site for the transcriptional activator. In the *in vitro* and *in vivo*, results indicate that the TAS-amplified bidirectional promoters greatly increased the expression level of the reporter gene. Therefore, this system might be useful when weak tissue-specific promoters are considered. An attractive feature of this system is that the compact size of such promoters enables accommodation of larger expression cassettes.

Dual promoter systems

Problems with IRES-driven gene expression have led to the development of vector constructs containing two promoters (Fig. 9E). Two independent, constitutive promoters were employed to enable co-expression of two genes in transduced engrafting HSPCs and their progeny, as well as in other human cell types (Yu *et al.,* 2003b). A similar system for reliable dual gene expression was described by Semple-Rowland *et al.* (2007). In their design two transgenes, each with its own tissue-specific promoter and arranged head-to-tail, were inserted into cHS4-insulated lentiviral backbone and shared the same bGHpA sequence that replaced the 3′ R/U5. Thus, the transcript encoded by the first gene was extended to include in its 3′ UTR the promoter and coding regions of the second gene followed by the insulator sequence that was placed in the U3 region to enable duplication of the cHS4 at the 5′ end of the provirus. The dual-promoter vectors used in this study were able to target expression of two genes to a single cell type as well as to two different cell types within the same retinal tissue. Depending on the combination of the promoters used, one gene can be expressed in one cell type and the other in another type of cells, proving flexibility of the system.

However, the two transcriptional units positioned in this tandem forward orientation suffered from low level and/or inconsistent expression, possibly due to promoter interference or suppression, often expressed in a cell type-dependent manner. In an elegant study on dual promoter-driven gene expression, Tian and Andreadis (2009) reported on the construction of lentiviral vectors in which two expression cassettes, inserted in a reverse orientation with respect to LTRs, were separated by polyadenylation, terminator and insulator sequences (Fig. 9F). The combination of those elements eliminated promoter interference yielding high-level gene expression similar to that obtained by single-gene-encoding vectors. Analysis of co-expression of two fluorescent markers (EGFP and DsRed2) led to the construction of optimized vectors that performed equally well with several different promoters and cell types tested. The best results were obtained when the first gene was terminated with an SPA synthetic polyA signal, a pause G-rich sequence from the extension of β-actin gene — T_{actb} terminator, and the cHS4 insulator. For optimal expression, the second ORF was followed by the thymidine kinase polyA (TKpA) signal and a synthetic sMAR8 insulator. Interestingly, some polyA sequences, e.g., SV40-derived one, reduced the viral titers significantly even when inserted in

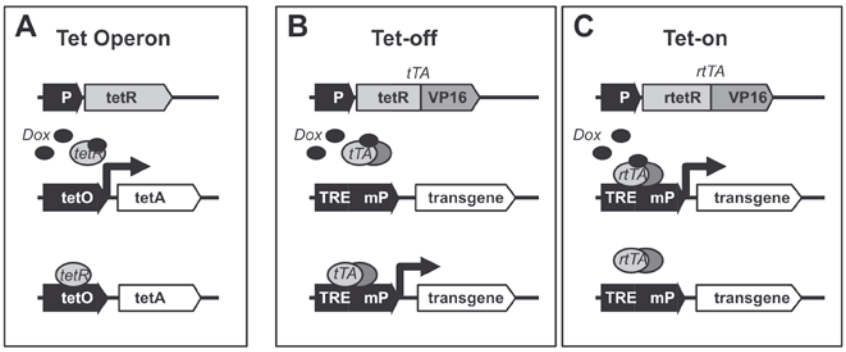

Figure 10. Tet operon-based system for regulatable gene expression from lentiviral vectors.
A. Bacterial Tet-resistance operon. In the presence of antibiotic, Tet-controlled repressor (tetR) dissociates from operator (tetO) and tetracycline resistance gene (tetA) is transcribed. **B**. Tet-off system. In the absence of doxycycline (Dox) Tet-controlled transactivator (tTA, with HSV VP16 transactivation domain fused to tetR) binds TRE promoter (several tetO elements upstream of minimal promoter) and activates transcription. **C**. Tet-on system. In the presence of Dox reverse Tet-controlled transactivator (rtTA, mutant version of tTA) binds TRE promoter and activates transcription.
Abbreviations: P, promoter; TRE, Tet-responsive element; mP, minimal promoter.

reverse orientation, possibly due to their ability to terminate transcription in both directions.

In summary, the expression of two or more genes from one vector was demonstrated using various methods. The multiple gene expression was achieved either from single, polycistronic transcripts (with the IRES and 2A peptide sequences) or by generation of the independent transcription units (using splicing mechanism or multiple promoters). Additionally, the combination of different methods can be successfully applied. Nonetheless, each approach has its specific strengths and limitations, and these advantages and weaknesses must be considered when designing the vector for the particular needs.

REGULATABLE EXPRESSION OF GENETIC INFORMATION

For effective gene therapy both efficient delivery of therapeutic genes to the target cells and their regulatable expression are desired. The earliest regulatable systems, based on natural inducible promoters, were disappointing because of a high basal expression, weak induction levels, and pleiotropic effects often exerted by their control elements on mammalian cells. To overcome those limitations chimeric regulatable systems were introduced. This novel approach utilized various prokaryotic and eukaryotic elements incorporated into promoters and offered better control of the transgene than could be achieved with natural inducible promoters. To date, several systems for gene expression in mammalian cells have been described that use exogenous drugs, including antibiotics, steroid hormones, and dimerizers, to control transgene expression (reviewed in: Agha-Mohammadi & Lotze, 2000; Clackson, 2000; Gossen & Bujard, 2002; Toniatti *et al.*, 2004; Goverdhana *et al.*, 2005). However, many of those systems still show either leakiness (i.e., undesired transgene expression when the inducer is absent) or poor activation (i.e., insufficient transgene expression in the presence of the inducer) and, in order to alleviate these problems, further improvement of the regulatable systems is a must.

Optimized tetracycline-responsive promoters

Among other systems, tetracycline (Tet)-responsive promoters have the most promising characteristics and the Tet-based systems are most widely used in lentiviral vectors. The system is entirely prokaryotic and employs components of *E. coli* Tet-resistance operon (derived from transposon Tn10) (Fig. 10A) (for review see Corbel & Rossi, 2002). Due to this, elements of the Tet-based system should not exert pleiotropic effects in eukaryotic cells. Another advantage of using Tet-responsive promoters is that their effector molecule, Tet, or its synthetic derivative, doxycycline (Dox), can be administered orally and are able to penetrate all tissues (also pass the blood/brain barrier). Importantly, Dox functions in the Tet-based system at levels below those required for bacteriostatic activity and has a relatively short half-life, which allows rapid response following withdrawal of the antibiotic (Baron & Bujard, 2000). This response does not exhibit a threshold characteristic and is dose-dependent (Gossen & Bujard, 1992; Gossen *et al.*, 1995; Kringstein *et al.*, 1998).

In the original Tet-off system developed by Bujard and co-workers (Gossen & Bujard, 1992), a Tet-controlled transactivator (tTA) binds the Tet-responsive element (TRE) and induces gene expression in the absence of Tet or Dox (Fig. 10B). The TRE consists of seven tandem copies of Tet operator (tetO) sequences (each 18 bp long) and is placed upstream of the CMV minimal promoter (TRE/CMV). tTA is a chimeric protein in which bacterial Tet re-

pressor (tetR) is fused to the HSV VP16 transactivation domain. In contrast, in the Tet-on system, the binding of the reverse Tet-controlled transactivator (rtTA), a mutated version of tTA, and the resultant gene expression is induced by Dox (Fig. 10C) (Gossen *et al.,* 1995). Hence, both Tet-based systems require that two components, an inducible transgene and the transactivator, be present in the same cell.

Several groups described inducible Lentivirus vectors based on the Tet-off system (Kafri *et al.,* 2000; Vigna *et al.,* 2002; Régulier *et al.,* 2002; 2003). Their results show very high induction levels — more than 500-fold above background. Unfortunately, these background levels in the repressed state were also unacceptably high. Nonetheless, the main limitation of this system, especially in clinical settings, is that constant presence of Dox is required to suppress transgene expression. In contrast, the Tet-on system requires the presence of the drug only to activate gene expression. Reiser *et al.* (2000) and Johansen *et al.* (2002) designed HIV-1-based Tet-on vectors involving the original rtTA. However, background expression levels were high with this system, which could be, at least partially, attributed to transcription from the 5′ LTR or cryptic promoters present in the neighboring chromatin. Moreover, transcription activation after addition of the inducer was poor.

Further attempts to improve Tet-inducible vectors have been made over the years. Koponen *et al.* (2003) used a SIN Lentivirus (133 bp deletion in U3 region) bearing a transgene controlled by a minimal CMV promoter coupled with TRE in combination with a second vector encoding enhanced rtTA2^{S}-M2 transactivator (Urlinger *et al.,* 2000). As one could expect, this transactivator performed much better than the original rtTA (130-fold induction), possibly due to its improved stability and enhanced sensitivity to Dox. However, the high background expression persisted (Koponen *et al.,* 2003).

Pluta *et al.* (2005) tested several different approaches toward reduction of the system leakiness and improvement of activation. First, direct comparison of the performance of various transactivators confirmed the superiority of rtTA2^{S}-M2 over rtTA, rTE4d38, and rtTA2^{S}-S2. Second, to minimize background expression, as much as 400 bp was deleted from the U3 sequence (ΔU3) present in the proviral LTRs. Third, a 1.2 kb-long cHS4 was incorporated in place of the U3 deletion in the 3′ LTR in order to shield the expression cassette from the influence of surrounding host chromatin enhancers (after duplication of the cHS4-containing ΔU3 LTR). Additionally, to increase the level of activated transgene expression and to reduce background transcription second-generation TREs, in which the distances and positions of consecutive tetO sequence elements had been optimized, were used. One of those improved TREs, TRE/Tight, was commercially available (Clontech), the other one, TRE/Pitt had been described by Agha-Mohammadi *et al.* (2004). In TRE/Pitt, the central bases of eight tetO sequences are separated by 36 bp (3.5 helical turns) and the TATA box of the minimal CMV promoter is placed 10 bp (1 helical turn) downstream of the first tetO element. Deletion in the U3 region alone did not significantly reduce the background transgene expression and it was concluded that the regular TRE/CMV promoter in the context of lentiviral vectors is leaky in the absence of both Dox and transactivator. As expected, the insulator sequences flanking the regulatable expression cassette reduced expression in the absence of Dox, but the induction levels were not as high as without cHS4. In turn, replacing TRE/CMV with the second-generation TREs decreased the leakiness and improved activation. The lowered basal expression might be explained by the absence of functional sequences that are present between the heptameric tetO sequences in the original TRE (Rang & Will, 2000). Background expression levels were lower with TRE/Tight than with TRE/Pitt, but induced expression levels in the presence of Dox were much

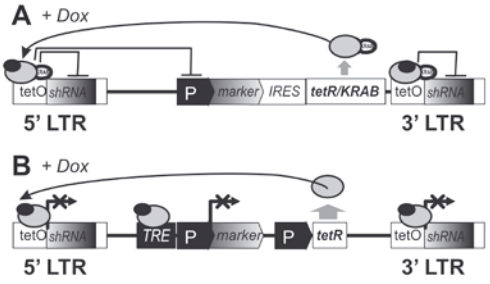

Figure 11. Single-vector system for controllable expression of shRNA and transgene.
A. System equipped with negative autoregulatory feedback loop. In repressed state (Dox present), Tet-controlled repressor, fused to KRAB protein silencer domain, binds to tetO sequences and blocks transcription of shRNA from RNAP III promoter. Simultaneously, due to KRAB chromatin silencing effect, transcription from internal RNAP II promoter, driving expression of marker gene and repressor (from IRES sequence), is also stopped. Thus, concentration of effector protein will decrease over time to a point where repression is partially relieved. Resulting re-synthesis of tetR-KRAB will repress system again. **B**. Simplified system based on bare tetR repressor. This system utilizes regular Tet-controlled repressor synthesized from independent constitutive promoter. Both regulatable promoters, RNAP III — driving synthesis of shRNA, and RNAP II for transgene expression, contain repressor-binding sequences. Continuous production of tetR ensures efficient repression of transcription in the presence of Dox.
Abbreviations: Dox, doxycycline; tetO, tetracycline operator sequence; shRNA, short hairpin RNA; P, internal promoter; IRES, internal ribosome entry sequence; TRE, Tet-responsive element; tetR, Tet-controlled repressor; tetR-KRAB, Tet-controlled repressor fused to KRAB protein silencer domain.

higher with TRE/Pitt than with TRE/Tight (522-fold and 90-fold induction, respectively). For the original TRE/CMV, induction was only 31-fold above background, whereas leakiness was 2.4 and 5.4 times higher than for TRE/Pitt and TRE/Tight, respectively. This regulatable system was then successfully tested *in vivo* (Pluta *et al.*, 2005).

Since binary vector systems were used for Tet-controlled gene expression, the undesired basal expression might be also attributed to the fact that the regulatory protein was not present in all cells. This problem can be bypassed by selection (sorting) of doubly transduced cells, but for *in vivo* studies this option is not available. The TRE/Tight promoter has been recently successfully used for construction of a regulatable single-vector system and this Tet-off platform (based on a modified tTA) was applied for an *in vivo* study in rat neurons (Hioki *et al.*, 2009). The principle of the design was that the marker gene (with its polyA signal) was placed under the control of TRE in reverse orientation and the transactivator was expressed from a tissue-specific promoter in forward orientation. This system supported a 40-times higher expression of the marker gene in neurons *in vivo* compared to a regular lentiviral vector (without the TRE promoter and in the absence of tTA). Moreover, the delivery of the transgene to target cells was about 7–8 times more efficient when the combined vector was used instead of its two-vector counterpart.

Regulatable expression of siRNAs

Another approach to controlling transgene expression using a Tet-based system employs Tet-controlled transcriptional repressors (tTS). In tTS tetR sequences were fused to the Krüppel-associated box (KRAB) protein domain of human KOX1, a Krüppel-type zinc finger factor (Deuschle *et al.*, 1995; Moosmann *et al.*, 1997). KRAB is an approximately 75-amino acid transcriptional repression module which can suppress both polymerase II- and polymerase III (RNAP II and RNAP III)-mediated transcription within a distance of 2–3 kb from its binding site, presumably by triggering the formation of heterochromatin (Margolin *et al.*, 1994; Senatore *et al.*, 1999; Ryan *et al.*, 1999).

Wiznerowicz and Trono (2003) used this system for conditional co-expression of a marker gene and small interfering RNA (siRNA) from a lentiviral vector (for recent reviews on RNA interference pathway, RNAi, and lentiviral vectors as a delivery tool, see: Morris & Rossi, 2006; Scherr & Eder, 2007; Llano *et al.*, 2009; Miest *et al.*, 2009; Manjunath *et al.*, 2009). In their design, both the tetO site and the small hairpin RNA (shRNA) sequence driven by an RNAP III promoter, are located in the U3 region and are duplicated in the integrated provirus. This

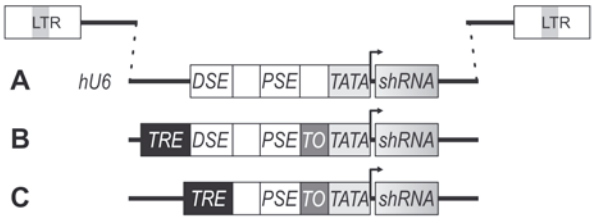

Figure 12. Adaptation of RNAP III promoter for regulatable shRNA expression from lentiviral vector.
A. Structure of human U6 promoter (hU6). **B**. hU6 promoter modified by insertion of single tetO operator sequence between PSE and TATA box and second-generation TRE sequence upstream of DSE. This construct facilitates tight expression control using doxycycline and tetR-KRAB repressor. **C**. DSE sequence of hU6 promoter, containing single tetO operator between PSE and TATA box, is replaced with second-generation TRE. This enables Tet-controlled activation of transcription with reverse transactivator bearing Sp1 transactivation domain.
Abbreviations: LTR, long terminal repeat; DSE, distal sequence element; PSE, proximal sequence element; TATA, TATA box; shRNA, short hairpin RNA; TO, tetO operator; TRE, Tet-responsive element.

allowed production of doubled amounts of siRNA in activated state (Dox present). In the absence of the effector tTS tightly suppressed expression of the shRNA from the promoter adjacent to the tetO site. However, with such a vector, where internal RNAP II promoter was placed further away from the tTS binding sites, leaky expression of the marker gene was observed (Zhou *et al.*, 2007). This could be avoided by placing TRE closer to the promoter. Optimized vectors were less leaky and activation of the system required only low concentrations of Dox (5 ng/ml). However, addition of Dox did not uniformly induce the transgene expression and "locked" suppression was observed in about 50% of transduced cells (Zhou *et al.*, 2007). This may suggest a serious limitation for the use of such chromatin-modulating suppressors.

Based on their original design (Wiznerowicz & Trono, 2003), Trono and co-workers reported an elegant single-vector system for controllable expression of shRNA and a transgene (Szulc *et al.*, 2006). The major modification was the insertion of an IRES/tTS cassette downstream of the marker gene (Fig. 11A). Thus, in the activated state, expression from the internal promoter leads to production of transgene, tTS and siRNA. In the repressed state, tTS binds to the tetO site near the RNAP III promoter/shRNA cassette in the vector LTRs and blocks transcription from both the RNAP III and the internal RNAP II promoter. The system comes in two flavors: Tet-on and Tet-off, depending on which tetR is used for creation of the fusion repressor (tTS or rtTS, respectively), so Dox can be used in both ways. Once the internal promoter is shut down, the concentration of

the effector decreases to a point when the repression is partially relieved. The resulting re-synthesis of tTS represses system again. This negative autoregulatory feedback loop (Fig. 11A) reduces the amount of tTS required for suppression. The system exhibited low leakiness in the repressed state and robust inducibility upon activation in both cell lines and primary cells as well as in mouse *in vivo* models.

Later, a similar system was described, albeit a much simpler one — without the autoregulatory features (Fig. 11B) (Zhang *et al.,* 2007a). The design was based on an unmodified tetR repressor expressed from a constitutive RNAP II promoter. Due to the lack of the KRAB silencer, tetR repressed only the tetO-equipped RNAP III promoter driving shRNA. Thus, the constantly high level of tetR ensured that the inducible promoter was effectively turned off. Moreover, this repression depended on simple TetR–RNAP III interactions, not on a chromatin remodeling mechanism. However, one possible limitation for such single-vector designs is the restricted payload of Lentivirus-based vectors. Also the proximity of the promoters, one driving the effector protein and the other — the gene of interest or siRNA, might result in transcriptional interference, therefore binary vector systems that separate these two expression cassettes are still popular.

Usually the RNAP III promoters, H1 or U6 (Fig. 12A), adopted to express siRNA in a Tet-regulatable manner, harbor a single tetO element inserted between the proximal sequence element (PSE) and TATA box, so it is tempting to introduce multiple tetO sequences in order to enhance the effect of regulatory protein binding. However, due to the very compact structure of the promoter such manipulations often lead to partial or complete abrogation of its function (Chen *et al.,* 2003; Lin *et al.,* 2004; Zhang *et al.,* 2007a). Other attempts to improve the performance of a regulatable RNAP III promoters were made by Pluta *et al.* (2007). In this case the human U6 promoter was engineered by insertion of a TRE sequence derived from the aforementioned TRE/Pitt promoter upstream of the distal sequence element (DSE). Therefore, the regulatable promoter contains a single tetO sequence between PSE and TATA plus additional eight tetO elements at the 5′ end (Fig. 12B). Using a SIN lentiviral system and the new Tet-responsive promoter the authors demonstrated a fully reversible decrease of expression of a biologically relevant target (CXCR4) to about 10% of a control experiment (absence of the siRNA) without any noticeable leakiness (no target gene silencing) in the absence of inducer (Dox). Notably, this robust silencing was achieved with relatively low multiplicities of infection (MOIs) (MOI of 25 for both tTS- and CXCR4 shRNA-expressing viruses) in a mixed cell population (unsorted cells).

In the same study the possibility of transactivation using modified RNAP III promoters was investigated. To make this "reverse" system, the DSE in the human U6 promoter, which is responsible for RNAP III transcription regulation (Murphy, 1997) was replaced with a second-generation TRE (Fig. 12C) in order to make it responsive to an Sp1-containing transactivator that preferentially transactivates RNAP III-type promoters (Das *et al.,* 1995; Strom *et al.,* 1996). In the presence of Dox, the modified rtTA with the VP16 domain replaced by an Sp1 domain was found to bind to the TRE and enhance transcription from the modified U6 promoter (Pluta *et al.,* 2007). Similar approaches have also been reported by others: an ecdysone-inducible hybrid RNAP III promoter combined with a GAL4/Oct2^{Q}(Q→A) transactivator (comprising the DNA binding domain of the yeast positive regulatory protein GAL4 and an artificial transactivation domain derived from the human transcription factor Oct-2) (Gupta *et al.,* 2004) or the TRE/U6 promoter responsive to rtTA/Oct2^{Q}(Q→A) (Amar *et al.,* 2006). However, all these interesting systems suffer from noticeable leakiness (unless transduced cells are sorted) and their fine-tuning requires more effort.

Tet-responsive RNAP II promoters have also been used for regulated siRNA expression. Stegmeier *et al.* (2005) described a system with Tet-regulated expression of a microRNA-like short hairpin RNA (miR-shRNA) using improved TRE promoters. Since microRNA-derived siRNA can be processed from longer precursors than regular shRNA sequences, it allows the use of RNAP II promoters for its transcription. In the presented design, miR-shRNA was expressed in a fusion with a transgene sequence or the marker gene was translated from an IRES following the miR-shRNA sequence. Hence, expression of the marker gene was only visible in cells in which siRNA-directed target gene silencing was induced. Although this system performed well at low MOIs (sorted cells), it relays on cells stably producing the transactivator.

More recently, a single-vector design that uses TRE to drive miR-shRNA has been reported (Shin *et al.,* 2006). In this construct the TRE/microRNA cassette is followed by a constitutive promoter driving expression of a bicistronic rtTA/IRES/marker gene cassette. Due to the constitutive expression of the transactivator and the reporter protein, all cells transduced with the all-in-one virus can be traced even when production of siRNA is not activated. Notably, this system is equipped with a positive feedback loop: in the activated state, translation of the longer transcript expressed from the TRE, containing also the rtTA and marker gene sequences, increases the amount of transactivator and amplifies the marker signal in cells with activated gene silencing.

Cre/loxP transcriptional switch

Another method for inducible siRNA expression is based on Cre/loxP recombination. This technique takes advantage of the ability of bacteriophage P1 Cre recombinase to delete from target DNA sequences flanked with two loxP sites. Cre-mediated excision of entire provirus (loxP sites inserted into the U3 region) has been used previously to turn off transgene expression at a desired time (Salmon *et al.,* 2000b; Mitta *et al.,* 2002; Chang & Zaiss, 2003). If such loxP-flanked sequences ("stuffer sequences") inserted between the promoter and shRNA or in the loop between the sense and antisense strands of shRNA, and hence hindering transcription, are removed, expression of functional siRNA begins. The Cre recombinase can be delivered to target cells by means of Lentivirus (Tiscornia *et al.,* 2004; Heldt *et al.,* 2007) or loxP/shRNA transgenic mice can be crossed with tissue-specific Cre-expressing mice (Ventura *et al.,* 2004; Yu & McMahon, 2006; Shukla *et al.,* 2007; Hitz *et al.,* 2007; Steuber-Buchberger *et al.,* 2008; Stern *et al.,* 2008; Hitz *et al.,* 2009).

Generally, because this mode of transcription activation is irreversible, such strategy is rather suited to study gene downregulation in transgenic animals, especially those genes whose permanent knockdown could result in embryonic lethality. Since many strains of cell- and tissue-specific Cre-expressing mice are currently available, this method is also useful for the analysis of temporal and tissue-specific effects of individual genes expression (Hitz *et al.,* 2007; Steuber-Buchberger *et al.,* 2008; Hitz *et al.,* 2009).

In summary, various systems for the regulatable gene expression have been used in a context of Lentivirus-based vectors. However, the Tet-responsive promoters are the most promising in terms of their high inducibility and low leakiness. Further improvements of the elements of this system should result in the creation of clinically relevant technology for the tight control of gene expression.

TISSUE-SPECIFIC PROMOTERS

Strong, ubiquitously active promoters, such as those of CMV, mouse phosphoglycerate kinase-1 (PGK-1), human translation elongation factor 1-α (EF1-α), human ubiquitin C (hUbC) or the hybrid CMV enhancer/chicken β-actin promoter (CAG), are widely used to express transgenes from integrated lentiviral vectors. Yet limitations of these promoters in the *in vivo* gene transfer have prompted a search for tissue-specific promoters and enhancers. Certain ubiquitously active promoters have recently been found to be silenced following transplantation of *ex vivo* transduced cells (Chang *et al.,* 2006; Zhang *et al.,* 2007b), in cloned feline embryos (Gómez *et al.,* 2009) and in human ES cells (Xia *et al.,* 2007). Moreover, the transgene expression driven by those promoters may provoke transgene-specific cellular and humoral immune responses, leading to the clearance of transduced cells from organs (Follenzi *et al.,* 2004; Limberis *et al.,* 2009).

One possible reason for this situation is unwanted transgene expression in antigen presenting cells (APCs), found in all tissues, when a viral vector is administered *in vivo*. It has been demonstrated that expression of the transgene (tumor antigen) in DCs leads to expansion and activation of antigen-specific T cells (Cui *et al.,* 2003). Moreover, Brown *et al.* (2007a) clearly showed that activation of IFNαβ is responsible for the rapid clearance of transduced cells from the liver. After *in vivo* administration of lentiviral vectors, induction of the type I interferon signaling response in DCs occurred within few hours and was independent of the envelope pseudotype. Instead, the activation of the innate antiviral response was concomitant with the presence of the reverse-transcribed vector DNA. Thus, before the development of an adaptive immune response against transgene transduction of hepatocytes was blocked at early stages of infection by induction of the IFNs.

Transcriptional targeting of the vector by means of tissue-specific promoters in combination with pseudotyping (see section on Targeting Lentiviral Vectors by Pseudotyping) in order to restrict transgene expression to desired cells and tissues provides a solution for some of the aforementioned problems. Examples of tissue-specific promoters and enhancers used to express transgenes from Lentivirus-based vectors are presented in Table 2.

While tissue-specific promoters used in a viral vector confer selectivity, their activity is usually weak and the level of transgene expression may be too low for detection or to exert the expected effect on the target cell. To address this issue, a CMV-derived enhancer was used in combination with several tissue-restricted promoters (Gruh *et al.,* 2008). This resulted in a multiple-log increase in marker gene expression compared to vectors lacking the enhancer, without, however, substantially compromising its tissue-specificity.

Another way to increase the level of tissue-specific expression is the transcriptional amplification strategy mentioned in the section dealing with Bidirectional Synthetic Promoters. While Liu *et al.* (2008) used the CMV promoter to drive the synthesis of a transactivator (GAL4BDp65; with the transcriptional activation domain of the NF-κB p65 protein) and a GAL4-responsive tissue-specific promoter to drive the reporter gene, Shaw *et al.* (2009) employed a reverse strategy for their TAS. In contrast to the design of Liu *et al.* (2008), the GAL4VP16 transactivator was transcribed from a tissue-specific promoter, whereas the gene of interest was placed under the control of a GAL4-responsive element. No

off-target expression was observed and the inducible promoter was able to increase the level of gene expression five–eight-fold over that obtainable with a standard promoter vector.

PRODUCTION OF LENTIVIRAL VECTORS — PRACTICAL GUIDE

The production and handling of Lentivirus-based vectors should be carried out with special attention paid to personal and environmental safety, using proper procedures and biosafety containment (i.e., Biosafety Level 2, BL2, containment; for more information see List of websites [10 and Appendix G-III-L,N therein]).

Protocols for production and concentration of lentiviral vectors are widely available in the literature. Successively modified procedures are regularly published by Reiser and co-workers (Reiser, 2000; Marino *et al.*, 2003; Kuroda *et al.*, 2009; Kutner *et al.*, 2009a; 2009b) and by others (Karolewski *et al.*,

Table 2. Selected tissue-specific promoters and enhancers used in Lentivirus-based vector context

Targeted tissue	Promoter	Source	Comments	References
retinal tissue	mouse CD44	gene encoding transmembrane glycoprotein and cell surface receptor for hyaluronic acid	active in glial Müller cells	Greenberg *et al.*, 2007
	human and mouse GFAP	gene encoding human and mouse glial fibrillary acidic protein	active in glial Müller cells	Greenberg *et al.*, 2007
	mouse VIM	gene encoding vimentin — major subunit protein of intermediate filaments	active in glial Müller cells	Greenberg *et al.*, 2007
	IRPB1783	gene encoding interphotoreceptor retinoid binding protein	active in cone cells	Semple-Rowland *et al.*, 2007
	GCAP292	gene encoding guanylate cyclase activating protein 1	active in cone cells	Semple-Rowland *et al.*, 2007
	mOP500	rhodopsin gene	active in rod cells	Semple-Rowland *et al.*, 2007
nervous tissue	CamKII	gene encoding calcium/calmodulin-dependent protein kinase II	active in neurons of adult forebrain	van Hooijdonk *et al.*, 2009
	SYN	synapsin 1 phosphoprotein gene	active in some regions of hippocampus	Kuroda *et al.*, 2008; van Hooijdonk *et al.*, 2009
	NSEp	gene encoding neuron-specific enolase	active in brain striatum and hippocampus	Lai & Brady, 2002
	$GfaABC_1D$	glial fibrillary acidic protein (GFAP) gene	active in glia in CNS	Liu *et al.*, 2008
β-cells	insulin	human insulin gene	active in β-cells and murine insulinoma cell lines	Shaw *et al.*, 2009
liver	ALB	albumin gene	active in hepatocytes	Follenzi *et al.*, 2004
	ET	synthetic promoter	more active than ALB; minor activity in spleen	Brown *et al.*, 2007
	ApoA-II	human apolipoprotein A-II gene	inducible by different factors, including fibrates, statins, etc.	Dagher *et al.*, 2009
	α1-AT	human α1-antitrypsin gene	active in hepatocyte progenitors and hepatocytes	Duan *et al.*, 2007

hematopoietic lineages	WASp	gene encoding Wiskott-Aldrich syndrome protein	active in T cells, B cells, dendritic cells (DCs) and CD34+ progenitor cells	Charrier *et al.*, 2007
	Tie2	gene encoding cell-surface angiopoietins receptor	active in endothelial cells (ECs) and monocytes	De Palma *et al.*, 2003; De Palma *et al.*, 2008
	promoters: ankyrin-1, α-spectrin, β-globin, ζ-globin enhancers: GATA-1, β-globin LCR, intron I8, α-globin HS40, γ-globin intron	erythroid-specific genes	active in erythroid lineages	Moreau-Gaudry *et al.*, 2001; Hanawa *et al.*, 2002
	HLA–DRα	gene encoding α subunit of human leukocyte antigen DR	active in antigen presenting cells (APCs) and DCs	Cui *et al.*, 2002
	proximal *lck*	gene encoding T-cell-specific protooncogene, *lck*	active in T cells	Lois *et al.*, 2002
heart	ANF	gene encoding human atrial natriuretic factor	active in cardiomyocytes	Gruh *et al.*, 2008
	MLC2v	gene encoding human ventricular myosin light chain	active in cardiomyocytes	Gruh *et al.*, 2008
lung	SP-C	type II alveolar epithelial cell (AT-2)-specific human surfactant protein C gene	active in AT-2 cells	Gruh *et al.*, 2008
prostate	PSAp	gene encoding prostate-specific antigen	–	Yu *et al.*, 2001; Zheng *et al.*, 2003
muscle	myogenin	–	–	Lois *et al.*, 2002
oviduct	OVA	chicken ovalbumin	marker gene exclusively expressed in transgenic hen oviduct	Lillico *et al.*, 2007

2003; Sena-Esteves *et al.*, 2004; Mitta *et al.*, 2005; al Yacoub *et al.*, 2007; Segura *et al.*, 2007; Ansorge *et al.*, 2009; Bagnis *et al.*, 2009). Moreover, a video tutorial for manufacturing retrovirus-based vectors by transient transfection of 293T cells can be found on-line (see List of websites [11]) (Gavrilescu & Van Etten, 2007). A schematic protocol for production of Lentivirus-based vector by transient transfection using DNA/calcium phosphate coprecipitation is presented in Table 3.

In this review, we will point out critical steps in the protocol and briefly describe recent advances in this field.

PRODUCTION OF LENTIVIRAL VECTORS BY TRANSIENT TRANSFECTION

The traditional method for lentiviral vector production relies on efficient cotransfection of producer cells, namely human embryonic kidney 293T (HEK 293T) cells (ATCC No. CRL-11268), with three (second-generation packaging system) or four (third-generation packaging system) plasmids: helper(s), envelope, and vector (Fig. 7; see section on Evolution of vector design concept).

Alternatively, viral vector production can be accomplished by transfer vector plasmid transfection of a producer cell line stably expressing packaging proteins and an envelope glycoprotein (reviewed by Cockrell & Kafri, 2007; see section on Envelope Expressing Cassette). This might reduce the risk of recombination between the transfected plasmids and minimize the problem of carrying over plasmid DNA in the vector preparation. However, this method is hampered by several limitations: the long time required to develop a stable cell line expressing all necessary vector components in a regulatable manner (due to possible cytotoxicity of some proteins, especially viral protease, Rev and VSV-G), the need for manufacturing designated cell lines for each desired vector pseudotype, low vector titers and, last but not least, genetic instability over long culture periods.

An interesting alternative for transient plasmid transfection is baculovirus-based Lentivirus

Table 3. Schematic protocol for production of Lentivirus-based vector by transient transfection using DNA/calcium phosphate coprecipitation

Day	Step	Operation
1st	plating cells	plate desired number of thoroughly separated HEK 293T cells (to get 50–80% confluence on the next day)
2nd (evening)	transfection	1. add chloroquine to culture medium (to 25 μM final concentration) 2. prepare transfection mix: a. mix water, plasmid DNAs and $CaCl_2$ (to 250 mM final concentration) b. add mixture drop-wise to equal volume of 2 × HBS pH 7.0–7.3 (fresh batch must always be tested for transfection efficiency) while vortexing (gently) 3. pipette DNA/calcium/HBS mix drop-wise onto cultured cells, swing plate to evenly distribute precipitate
3rd (morning)	culture maintenance	change medium
4th	transfection progress monitoring	check cells with fluorescence microscope: if transfer vector contains fluorescent marker gene — majority of cells should express it; if envelope vector codes for VSV-G — cell membranes should start to fuse
5th	collecting virus	harvest virus-containing medium, filter cell debris through 0.45 μm polysulfone filter, store at –80°C or concentrate directly

production (Lesch *et al.*, 2008). In this method, four recombinant baculoviruses deliver all necessary lentiviral vector components to infected 293T cells. This technique takes advantage of the fast and easy production of baculoviruses, efficient transduction of mammalian cells and the safety of baculoviruses that do not replicate in vertebrate cells.

Cell lines and plasmid DNA

The 293T cell line has some unique features that make it crucial for lentiviral vector production. The cells are highly transfectable (70–90% of positive cells) and additionally express SV40 T-large antigen, which enables replication of plasmids containing SV40 *ori* of replication (Soneoka *et al.*, 1995). The use of another HEK cell line derivative, 293E cells, expressing Epstein-Barr virus (EBV) nuclear antigen-1 (EBNA-1), has also been reported. These cells promote episomal persistence of plasmids carrying EBV *ori*P and can be used for large-scale transfection in suspension culture (Segura *et al.*, 2007).

The first critical point in viral vector manufacturing is the condition of the cell culture. The 293T cells should not be passaged more than 18–20 times before transfection. In the classical method, cells cultured as monolayers in medium supplemented with 10% fetal bovine serum (FBS) should be subconfluent (50–80% confluence) at the day of transfection (uniform distribution of plated cells is crucial). Production of lentiviral vectors by transient transfection of adherent cells growing in lower serum content or in serum-free media has also been achieved (Reiser, 2000; Geraerts *et al.*, 2005; Mitta *et al.*, 2005). The use of serum-free media helps to avoid the undesired influence of serum-derived contaminants on target cell infection. Some recently reported simplified methods use cells growing in suspension and without serum (Segura *et al.*, 2007; Kuroda *et al.*, 2009; Ansorge *et al.*, 2009) in order to reduce time, labor and cost of the procedure.

Another important issue with regard to transfection efficiency is the quality of plasmid DNA preparation. Plasmid DNA used for vector production must be free of bacteria-derived impurities (endotoxin free) and preferentially should be in a supercoiled form (scDNA). High yields of good quality DNA preparations are usually achieved using commercially available kits (Sigma-Aldrich, Promega, Invitrogen, Qiagen or equivalent plasmid DNA maxiprep kits). The total amount of DNA to be used for transfection (mixed plasmids) depends on the cell culture scale and usually ranges between 1 and 10 μg per 10^6 cells. In addition, a proper ratio of vector, helper(s) and envelope plasmid DNAs must be established. Typically, for the three-plasmid systems this ratio is 3:2:1, respectively (Kuroda *et al.*, 2009; Kutner *et al.*, 2009a; Kutner *et al.*, 2009b), and for four plasmids — 2:1:1:1 (Mitta *et al.*, 2005; Segura *et al.*, 2007).

Transient transfection

The routine and most efficient method for cells transfection is based on DNA/calcium phosphate (CaP) coprecipitation using HEPES-buffered saline (HBS) or BES-buffered saline (BBS) solutions. This procedure can be carried out on a small scale in multiwell plates (micro- or milliliters of medium) as well as on a large scale in big culture

vessels and bioreactors (liters of medium). Nonetheless, this procedure is labor-intensive and the reproducibility of results depends greatly on the pH of the buffer. Inconsistencies in pH may lead to significant batch-to-batch variations in titers. Therefore, samples of freshly prepared buffers that differ slightly in pH must be tested every time to get the most efficient transfection. Moreover, HBS is unstable and should be stored at –20°C. Additionally, the proper size of DNA/calcium phosphate crystals is of great importance for the transfection efficiency, which requires careful preparation of the mixture.

Hence, other methods have been used based on commercial lipid-based transfection reagents (FuGene, Roche; TransFectin, Bio-Rad; Lipofectamine, Invitrogen; etc). This technique offers good reproducibility, however, such reagents are more expensive than "homemade" solutions and are less efficient (Karolewski *et al.,* 2003). Nonetheless, manufacturers still search for potent transfection methods and new promising products appear on the market. For example, a new generation of transfection reagents has recently been released by Clontech (Xfect). These are polymer-based biodegradable nanoparticles that are claimed to facilitate high transfection efficiency in a wide variety of mammalian cell types. Similarly, new polyamine-based transfection reagents (*Trans*IT, Mirus Bio) outperform, as advertised by the manufacturer, lipid-based agents.

In order to develop a more reliable and cheaper method, several groups have tested transient poly(ethylene imine) (PEI)-based transfection (Geraerts *et al.,* 2005; Segura *et al.,* 2007; Kuroda *et al.,* 2009; Ansorge *et al.,* 2009; Bagnis *et al.,* 2009). This method is ideally suited for large-scale vector production, including cell factories or bioreactors (Geraerts *et al.,* 2005; Segura *et al.,* 2007; Ansorge *et al.,* 2009). PEI is relatively cheap and can be used in serum-free conditions. Moreover, PEI is chemically stable and ensures efficient transfection at a broad pH range. In direct comparison, the titers of vectors produced using PEI-mediated transfection are slightly lower than those obtained with the CaP-based method (Kuroda *et al.,* 2009), but the PEI-based vector production is more cost-effective and the protocol is shorter.

To enhance transient transfection efficiency, many protocols include addition of chloroquine (4-aminoquinoline) at the moment of DNA delivery (25 μM final concentration). This chemical is believed to reduce DNA degradation in lysosomes, but is toxic to the cells. It was shown that when used with PEI, chloroquine decreases viral titers when DMEM medium is used (Kuroda *et al.,* 2009). Thus, addition of sodium butyrate instead of chloroquine might be beneficial in some experimental settings (Karolewski *et al.,* 2003; Sena-Esteves *et al.,* 2004; Ansorge *et al.,* 2009).

The biochemical properties of the manufactured vector particles are different from those of the wild-type virus. For example, significantly fewer host cell proteins were found in lentiviral vector preparations than in HIV-1 virions (93 *vs*. 253) and the number as well as the composition of these proteins depends on the glycoprotein and transfer vector used (Wheeler *et al.,* 2007; Denard *et al.,* 2009). Moreover, only a small portion of the identified cellular proteins (25) was shared between HIV-1 and the lentiviral vector (Chertova *et al.,* 2006; Wheeler *et al.,* 2007). Nevertheless, as one might expect, the number of proteins present in vector and HIV preparations strongly depends on the purification conditions. Denard *et al.* (2009) estimated the number of possible virion-incorporated cellular proteins to as little as 23 while another 10 were found to co-purify with the vector. Among them, only 14 (or possibly even 9) were common for HIV-1 and lentiviral vectors, including syntenin-1, HSP70, HSC71, clathrin, actin, α-enolase, GAPDH, eEF1A, and ALIX.

In summary, lentiviral vector particles are usually manufactured by the vector plasmids cotransfection of producer cells (preferentially the HEK 293T cells). In order to achieve satisfactory viral titers all steps of the procedure must be tightly controlled. Additionally, the quality of DNA as well as proper cell culture status are crucial to get good results.

CONCENTRATION OF LENTIVIRAL VECTORS PREPARATIONS

Typical virus titers obtained from transient transfection range between 10^6 and 10^8 TU/ml of crude viral preparation and are sufficient for most *in vitro* studies based on transgene delivery. However, for gene silencing purposes or *in vivo* gene delivery, high multiplicities of infection (MOIs) in a limited volume are required. Thanks to the durability of pseudotyped viral vectors, increased titers can be achieved by physical concentration.

Notably, it is imperative that vector stocks to be used for clinical applications must be both concentrated and free of all impurities. Segura *et al.* (2006) published a detailed review on downstream processing procedures applied for retroviral vectors manufactured for gene therapy purposes.

The first clinical grade lentiviral vector, VRX496 (VIRxSYS Corporation), approved by the FDA's Cellular, Tissue, and Gene Therapies Advisory Committee (formerly called the Biological Response Modifiers Advisory Committee) for phase I pilot study, was manufactured by calcium phosphate

cotransfection of 293 cells with vector and helper plasmids (a two-plasmid system relying on expression of VSV-G from packaging vector, VIRPAC) in a 10-layer cell factory system. Vector-containing cell culture medium was collected at several time points after transfection. The bulk-harvested vector was filtered and concentrated by hollow fiber ultrafiltration. Contaminating nucleic acids were eliminated by treatment with benzonase. The vector preparation was finally purified by size-exclusion chromatography, and sterile filtered through a 0.22-μm filter (Slepushkin *et al.,* 2003; Lu *et al.,* 2004; Levine *et al.,* 2006).

Centrifugation

The simplest and the most popular method for concentration of vector preparations is ultracentrifugation (Naldini *et al.,* 1996a; Reiser, 2000; Sena-Esteves *et al.,* 2004; Kuroda *et al.,* 2009; Kutner *et al.,* 2009b). Virus-containing cell culture medium can be harvested two days after transfection or even one day later, if serum-free medium is used (Reiser, 2000; Geraerts *et al.,* 2006), and then is filtered using a 0.45-μm polysulfone filter. Viral particles are subsequently spun down for 1.5–2 h at 50–100.000 × *g* and 4°C. Resuspension of the pellet in a small volume of medium or buffer (e.g., phosphate-buffered saline, PBS) results in a more than hundredfold increase of the initial titer.

However, due to the shear forces generated during ultracentrifugation, this method is rather violent and may destroy certain vector pseudotypes. In such an event, centrifugation at a ten-fold reduced speed can be used to increase vector survival (Strang *et al.,* 2004). Additionally, vectors carrying large inserts in their backbones (above 7 kb) appeared to be particularly fragile and spinning at the reduced speed gave good virus recovery (al Yacoub *et al.,* 2007). Alternatively, VSV-G-pseudotyped viral particles can be precipitated (e.g., using poly-L-lysine or PEG) prior to low-speed centrifugation (Zhang *et al.,* 2001; Kutner *et al.,* 2009b). These techniques are particularly useful for processing large volumes of vector stock (liters of vector-containing culture medium).

In large-scale vector production, reduction of media volume before centrifugation is desired. Geraerts *et al.* (2005) reported the use of tangential flow filtration (TFF) prior to centrifugation, which allowed 66-fold concentration of initial preparation. In the next step, centrifugation gave a further 30-fold concentration.

Besides its roughness, ultracentrifugation may lead to problems with recovery of the vector. Pellets of viral particles produced in serum-free media are hard to resuspend and addition of 1 mM EDTA or 8 μg/ml protamine sulfate to the resuspension buffer might be necessary (Kuroda *et al.,* 2009). Another drawback of this method is that it concentrates all macromolecules present in the preparation (serum and host cell proteins, DNA), including inflammatory factors and potential viral infection inhibitors (reviewed by Segura *et al.,* 2006). Therefore, to roughly purify vector stock, virus-containing culture medium can be ultracentrifuged through a sucrose gradient (Baekelandt *et al.,* 2003) or cushion (Ricks *et al.,* 2008; Kuroda *et al.,* 2009).

Ultrafiltration

To bypass the problems outlined above, ultrafiltration can be used as an alternative (Reiser, 2000; Strang *et al.,* 2004; Sena-Esteves *et al.,* 2004; Segura *et al.,* 2007; Miyake *et al.,* 2007). For this purpose, Centricon Plus, Centriprep or Amicon 2000 and Amicon Ultra filters (all supplied by Millipore) with 50 or 100 kDa molecular mass cut-off membranes can be applied. These devices were initially developed for protein concentration and desalting. However, using ultrafiltration membranes, vector concentration comparable to that offered by ultracentrifugation and even better recovery are obtained with a substantially lower centrifugation force (2000–4000 × *g*) and shorter spinning time (15–30 min).

Additionally, this method enables removal of all contaminants smaller than the membrane pores. Indeed, Reiser (2000) observed vector titers much higher than expected from the achieved concentration factors and attributed this additional increase to the removal of putative transduction inhibitors.

Chromatographic purification

Unfortunately, ultrafiltration procedure also concentrates large host cell-derived contaminants which potentially could trigger immune response when vectors are used *in vivo*. Similarly to ultracentrifugation, another limitation of this method is its inability to handle large volumes of the preparation. Thus, for large-scale production of clinical-grade Lentivirus-based vectors, a scalable purification and concentration technique is highly desirable. Chromatography-based methods seem to be ideally suited to these requirements. Vector preparations have been concentrated and purified using size-exclusion chromatography (Slepushkin *et al.,* 2003), anion exchange chromatography (Yamada *et al.,* 2003) and heparin affinity chromatography (Segura *et al.,* 2007). However, all these methods give only about 50–70% recovery of the vector.

An interesting alternative method for simplified concentration and purification of lentiviral vectors by anion exchange chromatography is offered by Mustang Q Acrodiscs (Pall Corporation) (Marino *et al.,* 2003; Slepushkin *et al.,* 2003; Ricks *et al.,* 2008; Kutner *et al.,* 2009a; 2009b). The

membranes used in these units are strong anion exchangers with faster flow rates than achievable by traditional ion exchange resins, which makes the pre-concentration step dispensable. Reported yields of vector recovery exceed 75% (Kutner *et al.,* 2009a). Additionally, the Mustang Q Acrodiscs are designed for single use and can be adapted for both syringe bench-scale work and capsules for larger-volume applications (the capsule volumes range from 0.35 to 900 ml, enabling the processing of an estimated 1500 liters of vector medium per day). However, as for other chromatography resins, such membranes also bind serum- and cell-derived proteins as well as contaminating nucleic acids from crude vector preparations, and efficient vector recovery requires that low amounts of the impurities be present in culture medium (Kutner *et al.,* 2009a; 2009b). Moreover, it was also reported that vector stocks concentrated with this method are more sensitive to freeze/thawing (Kutner *et al.,* 2009a). This might be explained by a low content of protein impurities in the final preparation, since HIV-1-derived vector particles pseudotyped with VSV-G, have been shown to be particularly sensitive to freezing when harvested in serum-free media (Strang *et al.,* 2004).

Stability of purified vector particles

The sensitivity of vector stocks to freeze/thaw cycles is part of a larger problem — the thermolability of viral particles. In order to address this issue Strang *et al.* (2004) have tested the stability of different pseudotypes at 37°C. Regardless of the presence of serum, VSV-G-containing particles were stable up to six hours of incubation, whereas HIV-1 vectors with gamma-retrovirus envelope glycoproteins were partially inactivated already after two hours. Other studies showed that serum-free conditions during vector production had a negative impact on the stability of purified vector stocks. For example, it was observed that for gamma-retrovirus envelope pseudotypes the rapid decrease in infectivity at 37°C was a consequence of the inactivation of the reverse transcription process (Carmo *et al.,* 2009a). Since stability of vector particles produced for gene therapy applications is crucial, methods for protection of purified viral stocks are highly desired. It was recently demonstrated that addition of recombinant human serum albumin (rHSA) and lipoproteins to the storage buffer significantly increases stability of purified lentiviral vectors (Carmo *et al.,* 2009b).

In summary, the concentration and purification of a crude vector stock is necessary for most *in vivo* applications. Particularly, for the clinical grade vectors a multistep procedure is required. The method of choice for such vector preparation are chromatography-based techniques.

TITRATION OF LENTIVIRAL VECTOR STOCKS

A variety of quantification methods for vector titer assessment are in use (for review see: Segura *et al.,* 2006), each with specific advantages and disadvantages. Depending on particular needs, one titration method should be preferred over the others, taking into account that results can be either over- or underestimated. Hence, a combination of methods can be used in order to obtain the most reliable results. Protocols for the most often used titration methods are described in Kutner *et al.* (2009b).

The vector titration methods can be divided into two groups: 1) assays for assessment of the total number of vector particles present, and 2) functional titers, which correspond to the concentration of vector particles capable of transducing target cells. Although functional titers have greater importance in most applications, the ratio between the number of infective particles and the total number of vectors gives an idea about the preparation quality and helps monitor vector production reproducibility. In this section we will discuss differences in titers obtained using distinct methods, pointing at possible sources of errors.

Quantification of total number of vector particles

The total number of virus particles can be measured directly in a vector preparation by detection of vector components:

- p24 CA by enzyme-linked immunosorbent assay (ELISA);
- vector gRNA concentration in viral supernatant by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR);
- RT activity by product-enhanced reverse transcriptase assay.

These methods are relatively quick and may be conducted using commercially available kits (e.g., $p24^{gag}$ ELISA, Perkin-Elmer, and the RT assay, Roche). However, these techniques usually overestimate the functional vector titer. Quantification of p24 molecules does not discriminate between free CA and CA that originates from vector particles, including those that are non-functional. Moreover, titers obtained in immunoassay can be affected by the ratio of plasmids used for vector production, independently of vector specific infectivity (Logan *et al.,* 2004b). The qRT-PCR-based titers do not distinguish defective particles that contain gRNA. In turn, the RT-assay measures only the RT activity which can be different for distinct vectors.

Functional titers

The functional titer is usually evaluated by transduction of target cells with a serial dilution of the vector stock and is defined as the number

of transducing units per ml of virus stock (TU/ml). Quantification of the infective vector particles can be achieved by the following techniques:

- marker transgene expression assays;
- quantification of proviral DNA using quantitative PCR (qPCR);
- measurement of transgene mRNA levels by qRT-PCR.

Titration methods based on marker gene expression involve detection of fluorescent proteins (by FACS or fluorescence microscopy), immunodetection of non-fluorescent transgene product (also employing flow cytometry and microscopy), enzymatic assays (e.g., β-galactosidase assay for *lacZ* marker gene activity), antibiotic resistance of transduced cells (usually for selection genes that confer resistance to puromycin or G418/neomycin; in this case titer is defined as CFU/ml). These approaches are very useful when the assessment of vector infectibility is the goal. Yet, there are some disadvantages associated with the measurement of transgene expression. Transduction of target cells and synthesis of the marker protein takes several days, which makes the assay time-consuming. Quantification of infectious particles directly relies on transduction efficiency that is dependent on several factors, such as: time of cell exposure to the virus, vector sample volume, cell culture medium volume (rate of virus diffusion), etc (Zhang *et al.,* 2004b). Hence, these methods tend to underestimate the number of transduction competent vector particles. Indeed, in a direct comparison to proviral DNA-based method, EGFP expression-based titer was ten times lower. Even larger differences can be found if a weak promoter is used to drive the marker gene expression (Sastry *et al.,* 2002). In summary, although the use of some marker proteins (e.g., EGFP) may seem easy and straightforward, it requires careful consideration and proper planning of the experiment (see notes in Bagnis *et al.,* 2009).

Titers based on proviral DNA copy number are obtained using real-time qPCR and primers/probes targeting vector sequences present in most lentiviral vectors derived from HIV-1 (e.g., 5′ portion of *gag* or LTR). This method shares some limitations with the previous approach (i.e., transduction efficiency-dependence), but is independent of the marker gene or the promoter used. Nevertheless, DNA copy quantification can be affected by the limitations of PCR (e.g., accuracy of the standard curve). More importantly, the number of provirus integrations does not always correlate with the transgene expression level, due to chromosomal position effects (for references see section on Chromatin Insulators) and, thus, this approach may overestimate functional titers.

Therefore, the most accurate and reliable method for vector titration could be measurement of proviral mRNA levels by real-time qRT-PCR. This technique is marker expression-independent, yet measures real levels of transgene activity. It is particularly useful in the light of the fact that transgenes themselves can affect functional titers due to transgene-associated toxicity in both producer cells and target cells (Lizée *et al.,* 2003). Selection of the target mRNA sequence that is commonly used in Lentivirus-based vectors (e.g., WPRE) can simplify experimental design (Lizée *et al.,* 2003; Geraerts *et al.,* 2006).

Since choosing an optimal method for vector stock titration is a challenge, several research groups have conducted comparative studies on the accuracy of different approaches. As mentioned above, calculation of proviral DNA copy number is more accurate than tracking EGFP signal in a situation when the actual number of particles capable of transducing target cells is of interest (Sastry *et al.,* 2002). On the other hand, calculation of gRNA directly in virus supernatant gives overestimated titers. This can be partially attributed to the presence of background plasmids in the vector preparation, yet has little impact on protocols that include a transduction procedure and time for transgene expression (Sastry *et al.,* 2002). Although plasmid carryover is minimal during *ex vivo* transduction, it might be a problem for some titration methods and *in vivo* applications. Hence, to eliminate DNA contaminants vector preparations can be treated with benzonase (Sastry *et al.,* 2004) or DNaseI (Pluta *et al.,* 2005) prior to transduction.

In a comprehensive study, Geraerts *et al.* (2006) compared lentiviral vector titration methods that measure the amount of p24 (in pg/ml), gRNA (equivalents/ml), EGFP-based transducing units (TU/ml) and proviral mRNA equivalents. Not surprisingly, methods based on p24 concentration turned out to be the least reliable for the evaluation of functional vector particles. Still, this cheap and fast technique can be routinely used for quality control of virus production (TU per pg of p24). Similarly, gRNA content poorly correlates with functional titers and is rather dependent on the transfer vector features. In turn, fluorescence- and mRNA-based titers are very similar to each other and can be applied alternatively.

Transduction of target cells

Since the functional titer depends on transduction efficiency, constant infection conditions should be kept for vector stock testing. In this regard also selection of proper target cells is important. The HEK 293T, human osteosarcoma (HOS) (ATCC No. CRL-1543) and HeLa (ATCC No. CCL-2) cell lines are widely used for this purpose. All these cells are permissive to transduction, yet titers obtained in HeLa are usually lower (Kutner *et al.,* 2009a). Although the traditional reporter gene expression assay uses

adherent cells, employment of suspension-growing target cells, 293E cell line, was also reported (Segura *et al.*, 2007). Because the overnight cell attachment and trypsinization steps are skipped in this protocol, the time and complexity of the assay are reduced.

For routine vector titer evaluation, reproducibility of transduction is more important than its efficiency. However, for research applications, especially when cell types that are hard to infect are the subject, any trick that can boost transduction is highly desired. Generally, since vector particles must diffuse in the medium to reach the target cells and only the Brownian motion promotes the contacts between viral particles and target cells, the total volume of medium covering the cells should be minimal. Additionally, prolonged incubation with the virus or delivery of the vector at the same MOI in only half of the volume significantly elevates titers.

The majority of currently used protocols include addition of polybrene (hexadimethrine bromide) into medium during transduction (at a final concentration of 8 μg/ml). This polycationic agent is believed to promote virus-cell interactions by decreasing putative repulsive forces. The increase of vector titer after polybrene treatment can be six-fold (O'Doherty *et al.*, 2000). However, this positive effect is envelope-dependent and a comparative study of different pseudotypes showed that in the case of VSV-G the presence of polybrene did not substantially enhance the lentiviral vector titer (Strang *et al.*, 2004).

Since the infection efficacy depends on the rate at which the vector can reach target cells, methods for sedimenting viral particles during transduction might be helpful. According to this assumption, low-speed centrifugation (1200 × *g* for 2 h) of the cell culture plate following virus application greatly enhanced transduction (up to 45-fold) (O'Doherty *et al.*, 2000). This technique, referred to as spinoculation, simply accelerates the binding of viruses to target cells. However, similarly to polybrene, spinoculation increases the infection efficiency in a pseudotype-dependent manner (Strang *et al.*, 2004). As it was shown, for VSV-G-enveloped particles centrifugation gave less pronounced results (O'Doherty *et al.*, 2000; Strang *et al.*, 2004). Nevertheless, as one might expect, usage of polybrene and spinoculation simultaneously had an additive effect (Strang *et al.*, 2004).

An adsorption of viral vectors to cells can be also enhanced by nanoparticles. Hofmann *et al.* (2009) reported successful transduction of various cell types under nonpermissive conditions with lentiviral vector particles coupled to magnetic nanoparticles (MNPs). MNP-assisted infection, under such cumbersome conditions as the presence of hydrodynamic forces (shaking of the culture plate) and lowered temperature, was efficiently facilitated by the magnets placed under the plate. Interestingly, this technique also enabled targeting of vector/MNP complexes and positioning of transduced cells in desired organs and tissues when vectors and cells were administered systemically and then mice were exposed to a magnetic field.

For vector-based gene delivery to extremely sensitive targets, such as HSPCs, minimal *ex vivo* manipulation combined with high MOIs are required. Hence, protocols for infection of these cells employ conditions that do not alter their proliferation potential or ability to maintain multilineage differentiation. Lentiviral vectors have a greater potential than gamma-retrovirus-based vectors to transduce hematopoietic stem cells since efficient gene transfer with the former virus requires shorter time of incubation and little (or no) cytokine stimulation (De Palma *et al.*, 2003). Recently, application of retronectin (TaKaRa Bio), a recombinant human fibronectin fragment CH-296, for Lentivirus vector-based transduction has been reported (Kurre *et al.*, 2006; Lee *et al.*, 2009; Millington *et al.*, 2009). Retronectin facilitates co-localization of the virus particles and target cells. Although enhancement of HSPCs infection in the presence of this peptide is rather poor (two–four-fold), immobilized retronectin exerts a positive effect on the behavior of hematopoietic stem cells in *ex vivo* culture (retronectin-coated culture plates). A further increase in gene delivery efficiency can be obtained by combination of retronectin and spinoculation (Millington *et al.*, 2009). Interestingly, when mixed with the vector sample, retronectin also increases marker gene expression in bone marrow cells after direct bone marrow injection (Lee *et al.*, 2009).

Since the HIV-1 Tat protein shares some functional domains with fibronectin, its influence on infection efficiency has been investigated recently (Nappi *et al.*, 2009). Indeed, immobilized Tat, as well as Tat-derived peptides, bound viral particles and facilitated transduction with input doses that were otherwise insufficient to infect target cells. In contrast to the transduction process itself, binding of the virus to Tat appeared to be Env-independent. Another interesting observation is that, although Tat adsorbs viruses within a region responsible for its transactivation activity, Tat-mediated transduction enhancement does not require transactivation.

In summary, different vector titration methods have the specific strengths and weaknesses. Some of the methods tend to underestimate the number of infectious particles, other — to give overestimated results. Hence, the choice of the way to quantify vector particles depends on the particular needs and experimental setup. For example, FACS analysis of the cells expressing fluorescent marker can be used if transgene expression is of interest. On the other hand, measurement of proviral mRNA levels by real-time qRT-PCR is useful when the marker gene expression

is not planned. Finally, since the methods estimating functional titers are based on the cell transduction, reproducibility of infection procedure is crucial.

APPLICATIONS OF LENTIVIRAL VECTORS

During the last decade, lentiviral vectors have emerged as popular gene delivery tools in many areas of basic and applied research. An ever increasing number of scientific publications provides new facts about its flexibility and versatility. Here, we will give a brief outline of the state of the art of Lentivirus-derived vector applications.

APPLICATIONS OF LENTIVIRAL VECTORS FOR GENOME-WIDE FUNCTIONAL STUDIES OF GENE EXPRESSION

The ability to study genes function on a genome-wide scale is of tremendous value since it enables rapid identification of the major regulators of biological pathways and, thus, provide a global view of mammalian genetic circuits.

Recently, a combination of Lentivirus-based vectors and RNAi technology has led to the establishment of libraries for high-throughput loss-of-function screens in a wide range of mammalian cell types, including primary and nondividing cells (Root *et al.*, 2006). The reported libraries currently contain over 135000 lentiviral clones targeting 27000 human and mouse genes with multiple sequence-verified constructs. Using this technique, it was possible to identify more than one hundred putative regulators of mitotic progression and proliferation in human cancer cells (Moffat *et al.*, 2006) or to find kinases, whose silencing modulates sensitivity of tumor cells to an inhibitor of the mitotic spindle protein kinesin-5 (kinesin-5i) (Klinghoffer *et al.*, 2008). In a similar approach, screening of the arrayed lentivirus library expressing shRNAs targeting over 5000 human druggable genes for silencing events that activate β-catenin pathway identified dihydrofolate reductase (DHFR) as a novel modulator of the β-catenin and GSK3 signaling (Klinghoffer *et al.*, 2009). Reverse genetic screens showed their potential also in studies of the hematopoietic system. Using a lentiviral shRNA library, Ali *et al.* (2009) identified novel specific gene targets capable of altering HSPCs differentiation as well as shRNA constructs affecting stem cell expansion.

An interesting system for genome-wide studies of gene expression on the protein level has been described by Bialkowska *et al.* (2005). They presented genetic tool for protein tagging in mammalian cells based on lentiviral vector that harbors an artificial exon encoding EGFP tag. According to the preference of Lentivirus to integrate into transcriptionally active regions of host genome insertion of the artificial exon within introns of cellular genes resulted in expression of hybrid proteins consisting of the tag sequence fused in-frame to sequences of a cellular protein. Importantly, the tagged proteins were expressed from their endogenous promoters and the EGFP tag did not affect their subcellular localization. This technique enabled identification of tagged proteins by sequencing of RACE (rapid amplification of cDNAs ends) — PCR products and investigation of their subcellular distribution under variable physiological conditions using confocal microscopy. The protein tagging strategy could be also used for protein purification and studies on the protein–protein interactions.

LENTIVIRAL VECTORS IN ANIMAL TRANSGENESIS

The implementation of the Lentivirus-based gene delivery system in animal transgenesis has been discussed in detail by others (Park, 2007; Singer & Verma, 2008; Pfeifer & Hofmann, 2009). It is noteworthy that besides transgenic mice, rats, pigs, chickens and cows (see references in Park, 2007), also less popular species of transgenic cloned animals were generated recently using lentiviruses such as domestic cats (Gómez *et al.*, 2009) and prairie voles (Donaldson *et al.*, 2009). Genetic-modified animals are created by infection of fertilized or unfertilized oocytes, single-cell embryos, early blastocysts, embryonic stem cells or by transduction of cells that are used as donors of nucleus for somatic cell nuclear transfer (SCNT). Importantly, the progeny of these animals inherit and express the transgene.

Although lentiviral vectors have a smaller payload than is achievable with DNA-based methods, gene delivery *via* DNA microinjection displays a relatively low efficacy. Moreover, lentiviral-modified embryos show very high transgene expression levels and better survival rate. Utilization of Lentivirus-based vectors enables generation of transgenic animals with a tissue-specific and/or conditional expression of the desired gene or gene silencing. Therefore, this technique offers a good alternative for efficient and low-cost animal transgenesis for research applications, agriculture, biotechnology and biomedicine. It is particularly important in the light of the fact that large animals could be modified to become organ donors (pigs) or living protein factories (cows). Last but not least, the progress in Lentivirus-based transgenesis opens a gate to creation of virtually all possible animal models of human diseases. Recently, successful generation of the lentiviral-modified transgenic primate model of Huntington's disease has been reported (Yang *et al.*, 2008d). Due to the close genetic, physiological and neuro-

logical similarities between humans and monkeys, non-human primates are ideal models for studying human physiology and diseases.

LENTIVIRAL VECTORS FOR CELL ENGINEERING

Lentivirus-based vectors have recently emerged as the convenient tool for reprogramming of somatic cells. Induced pluripotent stem (iPS) cells focus recent attention as the possible source of autologous stem cells for use in regenerative medicine. Pluripotency can be induced in differentiated murine and human cells (e.g., fibroblasts) by lentiviral transduction of four transcription factors: Oct4, Sox2, Klf4, and c-Myc, as was originally reported by Takahashi & Yamanaka (2006) who used gammaretroviral vector.

Brambrink *et al.* (2008) obtained iPS cells from mouse embryonic fibroblasts (MEFs) infected with four vectors expressing the transcription factors from Dox-inducible promoters. Interestingly, it was demonstrated that the generation of iPS cells required the ectopic expression of the transcription factors for a minimum of 12–16 days. Moreover, normal differentiation of already derived iPS cells required subsequent downregulation of the transgenes. Another group used similar Dox-inducible lentiviral system to generate human iPS cells from keratinocytes and found that these cells required only 10 days of expression of the reprogramming factors (Maherali *et al.,* 2008). Switching Dox-inducible gene expression off and again on, enabled pluripotent cells differentiation and subsequent generation of "secondary" iPS cells at a frequency much greater than the initial conversion (Hockemeyer *et al.,* 2008; Maherali *et al.,* 2008).

However, the use of multiple vectors for cell reprogramming results in the high numbers of genomic integrations in iPS cells which limits their use for therapeutic applications. Hence, several research groups (e.g., Sommer *et al.,* 2009; Carey *et al.,* 2009; Shao *et al.,* 2009) have independently demonstrated generation of iPS cells with single lentiviral vector expressing a "stem cell cassette" composed of all four transcription factors. This powerful, yet simple system, expressing four genes from a single transcript (using 2A peptide sequences or a combination of 2A peptide and IRES), allowed induction of pluripotency with a single viral integration. The next step toward the application of iPS technology for clinical purposes was the implementation of the Cre/loxP recombination system. After induction of pluripotency in adult skin fibroblast by their transduction with single polycistronic lentiviral vector, expression of the Cre recombinase in generated iPS cells resulted in deletion of the vector from the host DNA (Chang *et al.,* 2009).

Such lentiviral vector particles that induce pluripotency of transduced cells are commercially available (Allele Biotechnology).

LENTIVIRAL VECTORS IN CLINICAL GENE THERAPY APPLICATIONS

Potential gene therapy targets accessible to lentiviral vectors but out of reach for most other gene delivery agents can be found in the CNS, liver, heart, kidney, muscle, ocular tissue and pancreas. Successful preclinical studies have been carried out for treatment of Alzheimer's, Parkinson´s and Huntington´s diseases. Unlike the CNS or other organs, therapy of most genetic disorders linked to the hematopoietic system does not require *in vivo* administration of the vector and can be achieved by an *ex vivo* transduction and subsequent transplantation of isolated autologous HSPCs. Over the last decade, several research groups have reported successful correction of genetic disorders, including immunodeficiencies and hemoglobinopathies, in preclinical animal models (for references see: Wiznerowicz & Trono, 2005; Ralph *et al.,* 2006; Cockrell & Kafri, 2007; Neschadim *et al.,* 2007; Nanou & Azzouz, 2009).

The first clinical study using a lentiviral vector was approved in 2001 for anti-HIV therapy (MacGregor, 2001). This trial was performed to evaluate the safety and efficiency of a conditionally replicating HIV-1-derived vector delivering HIV-1 envelope antisense gene to CD4+ T cells. Of note, the design of the vector (transfer vector contains both LTRs) makes expression of the *env* antisense RNA Tat- and Rev-dependent and, thus, basal expression increases when wild-type HIV infects vector-containing cells. In four of five patients with chronic HIV infection enrolled in this study, immune function improved after infusion of 10^{10} *ex vivo* gene-modified autologous CD4+ T cells. The presented results showed an absence of RCLs and a lack of clonal outgrowths (potential precursors to insertional mutagenesis) after 21–36 months of observation (Levine *et al.,* 2006). Additionally, a high-throughput analysis of vector integration sites in *ex vivo* vector-transduced CD4+ T cells and in cells recovered at several time points after infusion clearly showed preference for active genes and epigenetic marks associated with active transcription units. There was also no indication that proliferation of the transduced cells after infusion resulted in any enrichment for integration sites in proximity of proto-oncogenes or within tumor suppressor genes (Wang *et al.,* 2009a). Altogether, these reports show the clinical usefulness of the T cell culture system and gene transfer using lentiviral vectors.

Since that time, twenty-one clinical trials with Lentivirus-based vectors have been initiated or are

currently under review (including eight new clinical trials opened in 2008 and two under revision in 2009). This number constitutes 1.4% of the total 1537 gene therapy clinical trials employing distinct gene delivery methods. To date (March 2009), all trials, including phase I/II studies, were based on *ex vivo* vector delivery. Eight of the trials were performed for the treatment of HIV infection, nine for treatment of genetic diseases (X-linked cerebral adrenoleukodystrophy, sickle cell anemia, β-thalassemia, Wiskott-Aldrich syndrome, mucopolysaccharidosis type VII, Fanconi anemia complementation group A, Parkinson's disease, X-SCID) and four trials were directed against various cancers (data provided by *J Gene Med*; see List of websites [5]).

There is also a growing interest in the development of lentiviral vector-based vaccines against various diseases, including HIV infection (reviewed by Lemiale & Korokhov, 2009). Just recently, nonintegrating Lentivirus-based vectors have been shown to confer in mice immunity against West Nile virus (WNV) (Coutant *et al.*, 2008), HBV (Karwacz *et al.*, 2009) and against tumor cells expressing the ovalbumin (OVA) antigen (Hu *et al.*, 2009b; Karwacz *et al.*, 2009). Another interesting application of vectors derived from Lentivirus is in wound healing. Badillo *et al.* (2007) demonstrated in an animal model that lentiviral-mediated overproduction of stromal-derived growth factor-1α (SDF-1α) is sufficient to correct the pathophysiologic abnormalities associated with diabetic wound healing.

CONCLUDING REMARKS

Molecular studies that were initiated more than two decades ago, have led to the development of efficient and safe gene transfer system that is being successfully used for a wide variety of sophisticated *in vitro* and *in vivo* experiments. This was made possible by the constantly expanding knowledge about the virus structure and biology that we have gained from the decades-lasting research. However, this knowledge is far from being complete.

Vectors that utilize lentiviral genomes are characterized by a broad range of targets, long-lasting expression, relatively high capacity and low toxicity. They are also easy to adopt to such interesting and potent techniques as RNAi technology and targeted regulatable gene expression. Vectors derived from lentiviruses have also demonstrated their great potential in the generation of transgenic animals expected to be extremely beneficial in modern agriculture, biotechnology and biomedicine.

Lentivirus-based methods of gene delivery have proven their indisputable superiority over simple retroviruses in safe modification of quiescent, non-stimulated hematopoietic progenitor cells *ex vivo*, potent delivery of genetic payload to neurons in the CNS and to other differentiated cells in distinct organs (liver, pancreas, etc). They also appeared to be more potent than non-viral gene delivery methods in different experimental settings (Dullaers *et al.*, 2004; Cao *et al.*, 2009; Kim *et al.*, 2009). Consequently, lentiviral vectors have been successfully used for treatment of various genetic deficiencies and physiological disorders in animal models. Results from preclinical studies have provided grounds for the application of these vectors in human gene therapy.

The stigma understandably associated with HIV-1-based vectors forced the tremendous progress in vector system development in terms of its safety and availability of a variety of standardized procedures and assays for vector evaluation. That led to the first clinical trial involving a lentiviral genome-based vector to be approved eight years ago. Although, to the best of our knowledge, a satisfactory result of a clinical trial is yet to be reported, with the search for a perfect vector ongoing, such a breakthrough report cannot be far.

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LIST OF WEBSITES

1. www.emea.europa.eu
2. www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm
3. www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.061.1.06.htm
4. www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=11632&keep=1&lvl=6&filter=genome_filter&p=genome
5. www.wiley.co.uk/genmed/clinical/
6. www.unaids.org/en/KnowledgeCentre/HIVData/GlobalReport/2008/2008_Global_report.asp
7. www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/2009/sequence2009.pdf
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