INSIGHTS INTO THE MECHANISMS OF HIV-1 *CIS* ELEMENTS AND *TRANS* FACTORS REQUIRED FOR RNA ENCAPSIDATION AND TRANSDUCTION

Adam Cockrell

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Genetics and Molecular Biology

Chapel Hill
2011

Approved by:

Tal Kafri, M.D, Ph.D

Mark Heise, Ph.D

Tom Hope, Ph.D

Jude Samulski, Ph.D

Ron Swanstrom, Ph.D

Lishan Su, Ph.D

ABSTRACT

ADAM COCKRELL: Insights into the Mechanisms of HIV-1 *Cis* Elements and *Trans* Factors Required For RNA Encapsidation and Transduction
(Under the direction of Tal Kafri, M.D, Ph.D)

The retroviral replication process is typically separated into early events of infection for the virus to enter a host cell, and late events that generate new viral particles. Encapsidation (late event) and early infection events are governed by several different *cis* elements located in the viral RNA, and viral, or cellular, *trans* factors (proteins). A number of viral encoded components have been identified to function in the encapsidation process, most notably the Gag polyprotein and genomic RNA *cis* elements in the 5' untranslated region (UTR). Although conventionally associated with nuclear export of HIV-1 RNA, there is also a burgeoning role for the Rev/RRE in the encapsidation process. Additionally, *cis* elements of the RNAs encapsidated into viral particles may influence different early stages of infection into host cells. This dissertation employs an innovative approach that affords separation of *cis* and *trans* viral components to investigate their independent, and combined, effects on encapsidation and early events of infection that will be referred to as transduction. HIV-1 *cis* elements were reconstructed in the context of heterologous RNAs to assess encapsidation and transduction functions.

This work demonstrates for the first time that the Rev/RRE system can augment heterologous RNA encapsidation independent of all *cis* elements from the 5' UTR. In fact,

the Rev/RRE system appears to be required for specific and efficient encapsidation into HIV-1 viral particles, a process more commonly associated with Gag recognition of the canonical packaging signal in the 5’ UTR. Premised on encapsidation of a heterologous RNA into HIV-1 viral particles, our findings define a functional HIV-1 packaging system as comprising the 5’ UTR *cis* elements, Gag, and the Rev/RRE system, in which the Rev/RRE system is required to make the RNA amenable to ensuing interactions between Gag and the canonical packaging signal for subsequent encapsidation. Lastly, we show that heterologous RNAs can conform to transduction properties commonly associated with HIV-1 viral particles. Furthermore, some heterologous RNAs exhibit an episomal profile in transduced cells that may have improved safety benefits over more conventional nonintegrating lentiviral vectors. These innovative vector systems may prove beneficial for therapeutic gene delivery to nondividing cells.

ACKNOWLEDGEMENTS

I would like to thank:

My advisor, Tal Kafri

Present, and past, Kafri lab members that I have had the pleasure to spend time with: Boris Kantor, Matthew Bayer, Ping Zhang, Jian Dong, Thipparat Suwanmanee, Xiangping Li, Brian Zeithaml, Hong Ma, Blair Roszell, Karin Haack, and Adam Richter

Past and present members of the Samulski lab.

Sausyty Hermreck, Cara Marlow, and Bob Duronio from the Curriculum

My Committee Members:

Mark Heise
Tom Hope
Jude Samulski
Ron Swanstrom
Lishan Su

My sincere gratitude and appreciation are extended to my wife, Dawn Bowles, and my son, Everett Cockrell. Also I would like to thank our family members Chucky, George, Pepper, and Licorice.

TABLE OF CONTENTS

LIST OF FIGURES

Figure:

LIST OF ABBREVIATIONS

cis element	viral nucleic acid element in the context of the genomic RNA discussed
trans factor	protein that acts on cis elements
Ψ	canonical cis packaging signal in genomic RNA
U3	unique region 3 of retroviral genome containing promoter elements
U5	unique region 5 of retroviral genome containing regulatory sequences
PBS	primer binding site
R	repeat region
PPT/cPPT	polypurine tract/central polypurine tract
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
293T	human embryonic kidney cells derived from clone 293 and overexpressing large T antigen
ELISA	enzyme-linked immunosorbant assay
FACS	fluorescence activated cell sorting/scanning
HIV	human immunodeficiency virus
MLV	murine leukemia virus
Gag	group specific antigen, structural polyprotein
Pol	enzymatic proteins
IN	integrase

RT	reverse transcriptase
Pro	protease
Rev	regulator of expression of virion proteins
RRE	Rev Response Element
UTR	untranslated region
FIV	feline immunodeficiency virus
EIAV	equine infectious anemia virus
GFP	green fluorescent protein
CMV	cytomegalovirus promoter
att	attachement sites for integration
in vitro	experiments in tissue culture
in vivo	experiments in animals
CRM1	chromosome region maintenance 1
NXT1	NTF2-related export protein 1
Tap	nuclear export protein that forms heterodimer with NXT1
Env	viral envelope protein
FLuc	firefly luciferase gene

Chapter 1

INTRODUCTION

General Mechanisms of Viral Assembly and Genome Packaging

The fitness of a virus is dependent upon the genetic material (DNA or RNA) that encodes structural and enzymatic proteins required to generate a viable viral particle capable of protecting and mobilizing the viral genome between cells within an organism, and more importantly between organisms. Viral fitness is a consequence of mutation rate of a viral genome and the rate at which mutations become fixed within a population (54), culminating in the selection of those mutations that are most beneficial at different steps of a virus's replication cycle, within a particular host cell environment. Assembly of viral particles from individual capsid proteins to encapsidate the viral genome is a fundamental property shared by all viruses. Different viruses that infect organisms from all three domains of life (eukaryote, bacteria, and archaea) have been proposed to be evolutionarily similar based on the utilization of a common capsid structure for viral assembly (85, 113), and viruses assessed by comparative genomics reveal similarities in assembly and genome packaging (112, 114).

Viruses have evolved two general strategies for packaging their genome into capsids (200). In the first strategy some viruses form capsids, and then package their DNA, or RNA, into the preformed capsids through the use of energy-dependent molecular motors (EDMM). The molecular motor strategy is predominantly associated with bacteriophages,

such as T4. Nonetheless, herpesviruses share an evolutionarily conserved capsid structure with tailed-bacteriophages (113), and have been shown to employ a similar EDMM-based genome packaging strategy (144). It is not known if adeno-associated viruses (AAV) share capsid protein structural similarities with bacteriophages, or herpesviruses, nonetheless a EDMM mechanism has been proposed to facilitate encapsidation of the single-stranded DNA (ssDNA) AAV genome into preformed capsid particles (205).

The second strategy is independent of a molecular motor, therefore may be considered an energy-independent encapsidation mechanism. The viral genome is selected from the milieu of host cell RNAs through interactions of the viral capsid proteins with specific *cis* recognition elements in the DNA, or RNA, genomes to form a capsid around the genome during the assembly process. This mechanism is common to a variety of eukaryotic viruses including single-stranded RNA (ssRNA) viruses (Retroviruses (49) and Sindbis virus (211) and references therein), double-stranded RNA (dsRNA) viruses (Rotavirus (143)), segmented RNA viruses (Rotavirus & Influenza A virus (90)), and double-stranded DNA (dsDNA) viruses (SV40 (185)). Mounting evidence indicates that these two mechanisms of encapsidation can be distinguished from each other by measuring the mechanical properties of individual viral particle capsids using atomic force microscopy (186). In general, molecular motors appear to be necessary to package genomic material into viral capsids that are more rigid, or have a higher stiffness value, such as many bacteriophage & herpesviruses (186). The stiffness of retroviral capsids for murine leukemia virus (MLV) and human immunodeficiency virus (HIV) were determined to be much lower (109, 110), consistent with an RNA encapsidation mechanism that is independent of an EDMM mechanism. Furthermore, differences in capsid stiffness between immature and mature MLV and HIV

viral particles may contribute to both encapsidation and subsequent infectivity of a host cell (109, 110). Retroviruses have evolved unique biological properties that facilitate optimal RNA packaging to ensure efficient transfer of the genome to a host cell.

Retroviral Replication and Genomic RNA Encapsidation

The *Retroviridae* family of viruses comprises the *Orthoretrovirinae* and *Spumaretrovirinae* subfamilies, which can be distinguished at various stages of the viral replication cycle (46). Nevertheless, the genomic organization (Fig. 1) comprising *gag* (structural proteins), *pro* (protease), *pol* (reverse transcriptase and integrase enzymatic proteins), and *env* gene, the process of reverse transcription (RNA→DNA→RNA→translation of structural & enzymatic proteins, Fig. 2), and packaging of full-length genomic RNA into nascent viral particles, are common to all retroviruses. The vast majority of retroviruses constitute the *Orthoretrovirinae* subfamily which is morphologically and biologically classified into the following genera: *Alpharetrovirus*, *Betaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Gammaretrovirus*, and *Lentivirus*. Although morphologically, and biologically, distinguishable, the viral particles have a number of common features (Fig. 3): i) an Env glycoprotein which is processed into the surface domain and transmembrane domain that is inserted into an outer envelope (lipid bilayer obtained from a host cell membrane); ii) a Gag polyprotein that is processed into the matrix (MA) protein just inside the envelope which surrounds the capsid (CA) protein that forms the protein shell protecting the two strands of genomic RNA lined with the nucleocapsid (NC) protein; and iii) a Pol polyprotein which is processed into enzymatic proteins including protease (PR), reverse transcriptase (RT), and integrase (IN) (46). After generation of the nascent, immature retroviral particle a series of protease cleavage events

process the Gag and Gag-Pro-Pol polyproteins into the individual aforementioned proteins which can be observed by electron microscopy as dramatic morphological changes that reveal condensation of the virion core structure, resulting in formation of an infectious viral particle.

The mature, nascent viral particle initiates infection through interaction of the envelope glycoproteins with host cell receptors on the plasma membrane (retroviral replication seen in Fig. 4) (for a comprehensive review see (46)). The viral envelope fuses with the host cell plasma membrane releasing the capsid, and its contents into the cytoplasm. The retroviral capsid goes through an uncoating process, and the replication complex reverse transcribes the genomic RNA into dsDNA forming a preintegration complex in the host cell cytoplasm. The preintegration complex enters the nucleus, and the viral genomic dsDNA can integrate into the host cell genome. Alternatively, DNA products of reverse transcription can result in the formation of episomal DNA forms that do not integrate, including: 2-LTR circles that form from non-homologous end joining of a linear viral DNA; 1-LTR circles that can be generated directly as a consequence of the reverse transcription process, or through homologous recombination of the viral LTR's; linear viral DNAs that are not competent for integration; and mutant viral DNAs that may be generated by self-integration (34, 46, 169, 203). Nonetheless, the primary source of a productive infection is traditionally associated with the integrated proviral DNA form. Proviral genomic DNA is transcribed into viral RNAs that are translated into structural and enzymatic proteins used to generate viral particles as described above. Retroviruses harbor a major splice donor at the 5' end of the genome to promote differential expression of viral RNAs that encode envelope glycoproteins, or viral accessory proteins (41). This is most evident in lentiviruses that

encode a number of accessory proteins critical for the viral life cycle. Importantly, the full-length genomic RNAs (gRNA) of all retroviruses are not only used as templates for generating structural and enzymatic proteins, but must also be encapsidated into newly formed viral particles. Therefore, each retrovirus should have a mechanism capable of distinguishing between viral encoded RNAs competent for translation, and those competent for packaging into newly forming viral particles.

Efforts to distinguish between RNAs competent for packaging, and those utilized for translation, have primarily focused on MLV, and primate lentiviruses including HIV-1 and HIV-2. At least three distinct mechanisms have been proposed to describe how retroviruses may accomplish this (10, 18, 26). In the case of MLV two separate pools of viral gRNAs may be partitioned in the cell such that one is used for encapsidation into newly forming viral particles and the other for translation (53, 122). For HIV-1 and HIV-2 it appears that a single pool of gRNA is generated for both translation and encapsidation; however data has indicated that the mechanisms may bifurcate in how the gRNAs achieve encapsidation (53). HIV-1 gRNA encapsidation is not dependent upon translation prior to encapsidation, whereas some evidence has indicated that HIV-2 may utilize a cotranslational packaging mechanism in which unspliced gRNA is captured upon translation of the Gag polyprotein (69, 101). A recent study, however, challenges this claim indicating that packaging of HIV-2 genomic RNA is very similar to that of HIV-1, excluding the potential for a cotranslational packaging mechanism (156). Furthermore, numerous studies demonstrate that MLV, HIV-1, & HIV-2, as well as other retroviruses, can be utilized as retroviral vectors, wherein a vector RNA (containing all *cis* packaging signals, but no genes encoding structural/enzymatic proteins), *gag/pol* genes (structural/enzymatic protein expression cassette lacking *cis* packaging

signals), and *env* gene (typically a heterologous glycoprotein commonly derived from vesicular stomatitis virus) are separated onto at least three independent expression cassettes to generate viral particles (42, 43) (Fig. 5). The Gag/Pol proteins act in *trans* with *cis* elements in the vector RNAs to promote packaging of the vector RNA into nascent viral particles, indicating that translation of the vector RNA is not necessary for packaging. Lastly, a cotranslational packaging mechanism does not explain how the Gag polyprotein can be generated in vast excess (~1500-2000 molecules per viral particle) but only support encapsidation of 2 molecules of gRNA. If a cotranslational mechanism were feasible there must be a mechanism that determines when and which Gag polyprotein selects its corresponding gRNA for encapsidation. Although lenitiviruses apparently utilize a single pool of gRNAs for packaging, alternative mechanisms may be more suitable for explaining the distinction between the translational and encapsidation fates of gRNAs, some of which will be elaborated upon below with regard to HIV-1.

HIV-1 *Cis* Elements and *Trans* Factors That Facilitate Efficient & Specific Genomic RNA Encapsidation

Specific & efficient encapsidation of viral RNA into HIV-1 particles requires the selection of viral RNA species from the milieu of host cell RNAs, necessitating several complex interactions between *cis* elements in the viral RNA genome and *trans* factors encoded by the virus, & host cell genome (41, 121). Following transcription of gRNA in the nucleus the RNA must exit the nucleus and traverse the cytoplasm to the plasma membrane where it is encapsidated into newly forming viral particles (97, 116) (Fig. 6). The fate of the gRNA may initially be determined in the nucleus through formation of ribonucleoprotein complexes

initiated by Rev interacting with the RRE *cis* elements in the RNA (41). The HIV-1 gRNA can be partitioned into different regions comprised of *cis* determinants that function at various stages of viral replication. Some of the most well defined *cis* elements are confined to the 5' untranslated region (UTR) (Fig. 7). The 5' UTR of the full-length gRNA is a ~350 nucleotide long region that harbors a series of stem-loop structures that contribute to nearly every aspect of the viral life cycle including reverse transcription, integration, transcription of new viral RNAs, viral RNA splicing, translation into viral proteins and encapsidation of gRNA. An innovative biochemical technology called SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension) was recently employed to examine the structure for first 10% of the genomic RNA comprising the entire 5' UTR *in virio* and *ex virio*, at the single nucleotide level (Fig. 8) (214), as well as the entire HIV-1 RNA structure (212). Similar *ex virio* and *in virio* studies also revealed the structural significance of MLV genomic RNA for recognition by the MLV Gag polyprotein and for subsequent encapsidation into viral particles (64, 65). These high resolution structures provide strong evidence for nucleocapsid-RNA, as well as intra- and intermolecular RNA-RNA interactions as they exist in the mature viral particle. Also of note, at this point in the viral particle maturation the Gag and Gag-Pol precursor polyproteins are processed into their constituent proteins. Therefore, protein-RNA and RNA-RNA interactions may impose RNA structures in the mature viral particles resulting in conformations that are vastly different than those acquired during the process of encapsidation in the host cell. RNA may encounter various RNA-RNA and protein-RNA interactions in the form of ribonucleoprotein complexes in the host cell that may impart alternative RNA structural variations in the cytoplasm, which ultimately promote efficient transport and, consequently, RNA encapsidation. It may be of interest to perform

similar SHAPE analysis on viral RNAs in ribonucleoprotein complexes isolated from producer cells. Currently, mutational and genetic analysis combined with traditional biochemical and bioinformatic approaches provide insight into protein-RNA and RNA-RNA interactions that impact RNA structure and function within packaging cells.

Nucleocapsid, in the context of Gag or Gag-Pol polyproteins, are the most common protein-viral RNA interactions known; nonetheless, particles can still assemble in the absence of NC (165). In the absence of RNA particle assembly cannot occur, making RNA the major constituent acting as a scaffold upon which subsequent Gag-Gag interactions assemble into viral particles (24, 27, 28, 35, 72, 127, 128, 150, 151). Viral and cellular RNAs can be utilized for retroviral particle assembly, as it is well established that retroviral particles package numerous cellular RNAs, especially in the absence of viral RNA (152, 161, 162, 188). Furthermore, HIV-1 viral particles can be generated *in vitro* from purified Gag polyproteins only in the presence of nucleic acids (28, 72). The NC-viral RNA interaction provides a selective advantage for encapsidating viral RNA into nascent particles. However, in the absence of HIV-1 NC viral RNA is not selectively packaged, but rather cellular RNAs can be used as the scaffold and a determinant in the matrix (MA) protein of Gag was identified as an additional RNA interacting partner, thereby facilitating viral particle assembly (165, 166). Mutating both NC and the MA determinant resulted in loss of HIV-1 particle assembly (166). Nucleocapsid and MA proteins can non-specifically interact with viral or cellular RNA in the absence of proper *cis* encapsidation signals in the gRNA.

Encapsidation signals in the genomic RNAs of lentiviruses (HIV-1, HIV-2, SIV, & FIV) and simple retroviruses (MLV) have been defined through biochemical, mutational and genetic analysis (4, 21, 36, 49, 63, 79, 103, 120, 134, 135, 138-140, 170, 183, 197). The

major encapsidation signals are localized to the 5' UTR of retroviruses. The conventional major *cis* packaging signal (ψ) for HIV-1 is a ~120 nucleotide long fragment comprised of four stem-loop structures located in the HIV-1 5' UTR, and extending into the 5' end of the HIV-1 Gag coding sequence (49) (Fig. 7). The HIV-1 Gag polyprotein ensures specific and efficient encapsidation of viral gRNA through high affinity interactions of zinc fingers in the nucleocapsid (NC) portion of the Gag polyprotein with stem-loops 2, 3, and 4 in the gRNA (49). In addition to its capacity to interact with NC stem-loop 2 contains the major splice donor sequence which promotes the generation of alternatively spliced viral RNAs that encode all the accessory proteins, as well as the envelope glycoprotein required for viral infectivity. Stem-loop 4 includes the AUG translation initiation codon for Gag, and has also been determined to form a stem structure in long-range interactions with a region in the 5' U5 (214) (Fig. 8), at least in the context of mature viral particles. Although HIV-1 nucleocapsid does not interact strongly with stem-loop 1 this region contains a palindrome sequence termed the dimerization initiation site (DIS) in the GC rich loop that initiates the non-covalent dimerization of two molecules of single-stranded viral RNA, commonly referred to as a "kissing" interaction, and is thought to have an essential role in the encapsidation of viral RNA species (49, 95, 148, 168). Even though a preponderance of studies suggest a central role for the DIS in encapsidation of gRNA (reviewed in (168)), evidence indicates that the mechanism is more elaborate than simply dimerization of two single-stranded RNA molecules. Mutation of the dimerization domain and the entire stem-loop 1 structure demonstrates that the DIS is important for replication in a T cell line, but not in PBMCs (84), indicating that cellular factors, and possibly additional viral factors can influence encapsidation. Importantly, stem-loop 1 is present at the 5' UTR of both full-

length genomic RNA, as well as subgenomic, spliced, HIV-1RNAs competent for translation. An earlier model posited that the genomic RNA could conform to two states that would distinguish between RNA competent for translation and RNA to be directed for packaging: i) a long-distance interaction (LDI) conformation in which the DIS is occluded in a stem was suggested to be amenable for translation but not encapsidation; and ii) a branched multiple hairpin (BMH) structure in which the DIS is available for dimerization and interaction with Gag for subsequent encapsidation (49, 168). In contrast to the RNA switch model, evidence indicates that both conformations can support translation (2), and that the BMH conformation is favorable in both cellular HIV-1 RNAs and virion RNAs (167). Moreover, if dimerization were part of a selection process that determined the encapsidation potential of viral RNA then it might be expected that only full-length gRNA would form dimers competent for encapsidation; however, an *in vitro* study demonstrated that subgenomic RNAs can also form dimers and heterodimers (192). Apparently the HIV-1 DIS, as well as additional *cis* elements, serves a dominant role in the encapsidation process; however, additional viral and cellular components may also contribute to efficient and specific gRNA encapsidation.

A *bona fide* packaging system could be considered one that confers an efficient and specific gain of encapsidation function upon a heterologous RNA. In the case of HIV-1 transfer of the ψ *cis* packaging element to a non-HIV-1 (heterologous) RNA did not confer encapsidation of the RNA into HIV-1 viral particles (15), indicating that HIV-1 ψ is not sufficient for encapsidation. In contrast, *cis* elements from MLV (~175 contiguous nucleotide region in the 5' UTR) can readily support retroviral RNA packaging if relocated to a different position within homologous RNA (134), and is sufficient for encapsidation of

heterologous RNAs into MLV viral particles (3, 83). The HIV-1 packaging signal is more complex than that of MLV, requiring additional *cis* elements outside the canonical packaging signal. Independent studies using mutational analysis and genetic manipulation demonstrated that *cis* elements within the HIV-1 5' R, U5, and PBS regions of the RNA may also function in gRNA encapsidation (37, 38, 81, 139). Heretofore, several HIV-1 inter- and intramolecular RNA-RNA, and protein-RNA, interactions were discussed as components of the RNA encapsidation mechanism. These various interactions are necessary cytoplasmic steps that prompt gRNA encapsidation at the plasma membrane. In order to present a gRNA amenable to RNA-RNA and protein-RNA interactions in the cytoplasm the gRNA must initially exit the nucleus after transcription and localize appropriately in the cytoplasm. Therefore, additional viral *cis* elements and *trans* factors may be required components of a packaging system that supports efficient and specific gRNA encapsidation.

HIV-1 Rev/RRE (Rev Response Element) System and Nuclear Export, Translation, & Encapsidation of Viral RNAs

HIV-1, as well as all lentiviruses, is considered a complex retrovirus because of additional accessory proteins required for a productive infection. Originally termed the *art* gene, Rev (regulator of expression of virion proteins) was identified as one of two accessory proteins, the other being Tat, which are absolutely required for transactivation of HIV-1 structural and enzymatic proteins (57, 195). Shortly thereafter a *cis* element within the *env* gene at the 3' end of the genomic RNA, termed the Rev response element (RRE), was identified as the cognate sequence that the Rev protein recognizes in the gRNA (39, 132, 160, 187, 224). The HIV-1 Rev/RRE interaction has since been proposed to function in a number of processes

associated with viral replication including splicing regulation, nuclear export of full-length and partially spliced viral RNAs (i.e. intron-containing HIV-1 RNAs), enhancing translation of viral proteins, blocking integration of viral particles as a result of superinfection, and facilitating encapsidation of genomic viral RNA (reviewed in (68, 71, 199)).

The RRE forms a series of stem-loops structures that is retained in both full-length HIV-1 gRNA and partially spliced viral RNAs, thereby making the RNA accessible to interaction with the Rev protein (Fig. 9) (176). Mutational mapping, combined with genetic analysis, revealed a number of functional domains in the Rev protein (86-88, 131, 133, 176). The most prominent feature of Rev is its ability to shuttle between the nucleus and cytoplasm. Rev protein has an arginine rich nuclear localization signal (NLS) that is involved in nuclear, and ultimately nucleolar, localization through interaction with importin-β (7, 82). Shortly after transcription, Rev interacts with RRE-containing viral RNAs through a functional domain that overlaps the arginine-rich nuclear import signal (87, 176). In order for Rev to interact with its cognate RRE, RNA harboring the RRE *cis* element must be stabilized and retained within the nucleus; and, potentially localized to a nucleolar region that supports a stable interaction between Rev and the RNA. Recognition of splice donor sequences by host U1 snRNA stabilizes RNA in the nucleus, even in the absence of active splicing, obviating RNA degradation (98, 124). Additionally, AU-rich *cis* repressor sequences (CRS), or instability (INS) elements, mapped primarily to RNA regions within *gag*, *pol*, and *env*, mediate RNA retention within the nucleus (40, 129, 153, 190). Genomic RNA and partially spliced subgenomic RNA harboring CRS elements were found to localize in nuclear regions similar to those of the Rev protein, but distinct from fully spliced transcripts; indicating a putative common locale for Rev and transcripts containing the RRE (16). Futhermore,

mutating the CRS elements allows for nuclear export of RNA, and subsequent protein expression, independent of the Rev/RRE system (66, 111). The interaction between Rev and the RRE may mask the NLS, thwarting nuclear import of Rev/viral RNA complexes so that Rev/RNA complexes can be efficienctly exported to the cytoplasm (199).

Nuclear export of Rev/RNA complexes is mediated through a nuclear export signal (NES) in a leucine-rich region of the Rev carboxy terminus (131, 133) (Fig. 9). Unique to the Rev protein is the capacity to facilitate nuclear export of incompletely spliced viral RNAs, thus the Rev/RRE interaction can alter the ratio of unspliced/spliced viral RNAs by interfering with splicing (31, 55, 57, 77, 107, 108). Nuclear export of incompletely spliced viral RNAs is achieved by Rev bridging an interaction between the viral RNA and host chromosome region maintenance 1 (CRM1) nuclear export pathway (58, 59, 155, 163). Few cellular RNA have been determined to be exported through the CRM1 nuclear export pathway (ribosomal RNAs, U1 snRNA, SRP RNAs and very few cellular mRNAs) (91), most of which appear to be dependent upon RNA POL I or POL III mechanisms of transcription and are in the context of ribonucleoprotein (RNP) complexes comprised of adaptor proteins that bridge interactions between the RNAs and the CRM1 protein. Similarly, it has been proposed that Rev and incompletely spliced viral RNAs are part of a RNP complex, since numerous host cell proteins also impact Rev-dependent nuclear export, as well as downstream viral function that can interfere with HIV-1 replication (41, 71, 199, 201).

Nuclear export is a fulcrum of posttranscriptional regulation shared among retroviruses. The Rev/RRE system is a highly conserved mechanism employed among lentiviruses (119, 132, 145, 164, 174), whereas nuclear export of viral RNAs encoded by

type D simian retroviruses (SRV-1 and MPMV) is accomplished through a *cis*-acting constitutive transport element (CTE) in the RNA (20, 226). Identified CTE's differ from the HIV-1 Rev/RRE system in that nuclear export is independent of both the CRM1 pathway and a virally encoded protein. The CTE recruits the cellular protein TAP/NXF1 to direct RNA out of the nucleus through the TAP/NXT1-dependent pathway (73). The TAP/NXT1 pathway is the most common pathway utilized for nuclear export of spliced cellular mRNAs, as well as fully spliced mRNAs encoding Tat, Rev, and Nef proteins. Of note, the TAP/NXT1 pathway may also be utilized by simple retroviruses such as MLV, which do not encode an accessory protein for nuclear export, but rely upon *cis* elements within the packaging signal (106, 193), which presumably interact with cellular factor(s). The fact that lentiviruses have evolved the use of a Rev/RRE based system for nuclear export specifically through the CRM1 pathway indicates that this pathway of nuclear export may determine the cytoplasmic fate of viral RNAs and impact downstream biological processes at late stages of viral replication. A number of studies have demonstrated that proper viral assembly is governed by the pathway that the gRNA is exported from the nucleus to the cytoplasm (93, 94, 149, 191, 201). These studies imply that the Rev/RRE system influences the assembly of nascent viral particles, and presumably may regulate virological functions that occur prior to (translation of viral RNAs), or coincident with (viral gRNA encapsidation) particle assembly.

Nuclear export of viral RNAs is the dominant posttranscriptional regulatory mechanism associated with the Rev/RRE system; however, mounting evidence demonstrates that the Rev/RRE system is more than a nuclear export system (71). Separating the nuclear export function of Rev from its impact on viral events in the cytoplasm, including viral RNA translation and encapsidation, has proved to be challenging. Rev mutants that impact

translation and encapsidation, but not nuclear export, have yet to be identified. Furthermore, using available Rev NLS mutants to restrict Rev function to the cytoplasm would also preclude interaction with the RRE-containing RNA in the nucleus, not to mention that mutants in the NLS may also directly impact its interaction with the RRE since these are overlapping functions. A well established method has been to fractionate cells into cytoplasmic and nuclear parts followed by RNA isolation, affording that ability to distinguish between Rev-dependent viral RNA nuclear export function and influences on translation or encapsidation. A number of reports indicate that Rev encourages the translation of RNAs with minimal impact on nuclear export (8, 9, 48, 70, 172). Evidence indicates that Rev augments polysome loading for ensuing translation, whereby translation is enhanced several hundred fold with a minimal increase in nuclear export of viral RNAs (48). Rev may influence translation through recruitment of cellular proteins as is evident from a recent study with the cellular methyltransferase protein, PIMT, which trimethylates the 5' RNA cap of some HIV-1 RNAs as a regulatory mechanism for selective expression (221). Importantly, this mechanism was shown to be Rev-dependent, whereby Rev recruits PIMT to RRE-containing RNAs. Interestingly, this study did not investigate the potential of encapsidating 5'-cap trimethylated viral RNAs. Plausibly, this could be part of a mechanism that distinguishes between Rev-dependent RNAs targeted for translation compared to Rev-dependent RNAs targeted for encapsidation.

Similar to translation, encapsidation is a measure of the ratio of RNA encapsidated into viral particles relative to RNA available for encapsidation in the cytoplasm, thereby accounting for any changes in nuclear export. Early indications that the Rev/RRE system may be important for encapsidation of viral RNAs implicated regions within the HIV-1 *env*

gene (181). Further mutational analysis of the *env* gene, however, indicated that although regions within the *env* gene are important for encapsidation, the RRE itself was not essential for encapsidation (102). Moreover, partially spliced (RRE positive) and fully spliced (RRE negative) viral RNAs were shown to package with equal efficiency, but both were significantly less efficient than full-length RNAs (89). In opposition to these studies the RRE-deficient HIV-1 vectors were demonstrated to have much lower titers and less RNA packaged than vectors containing the RRE (125), indicative of a severe RNA packaging deficiency since mutation of the RRE had minimal influence on RNA exported to the cytoplasm. In consonance with this study HIV-1 derived viral vectors showed increases in vector titers with nominal changes in cytoplasmic RNA levels (5). Along these same lines the Rev proteins of HIV-1 and SIV were demonstrated to enhance encapsidation of gRNA into HIV-1, or SIV, viral particles, respectively (19). Moreover, the same study showed that the RRE was not absolutely necessary, but rather interactions between Rev and the RNA needed to be maintained for the observed augmentation in gRNA encapsidation. In addition to the RRE, Rev was identified to interact with a determinant in stem-loop 1 of the *cis* packaging signal (62). Mutational analysis of this determinant in the innate viral context disrupted RNA trafficking, as well as RNA encapsidation (67). Recent *in vitro* translation studies demonstrated that the *cis* determinant in stem-loop 1 is also important for Rev-mediated translation of viral RNAs, and that the RRE is not important for this effect (70). Moreover, translation was enhanced only when using moderate concentrations of purified Rev protein, but inhibited using high Rev concentrations. Potentially, the Rev concentration may act as a "switch" between translation (moderate Rev concentrations) and encapsidation (high Rev concentrations). It may be that both the RRE and *cis* determinant in stem-loop 1 of the

packaging signal are important for encapsidation of gRNA. Regardless, the interaction of Rev with the gRNA is predominantly mediated through the RRE (and putatively, additional *cis* determinants), and the Rev protein may influence not only nuclear export, but also translation and, as will be investigate in the work shown here, gRNA encapsidation.

Introduction to Dissertation Research

Efficient and specific encapsidation into HIV-1 viral particles is a process in which the RNA must travel from sites of transcription in the nucleus to sites of particle assembly at the plasma membrane (Fig. 6). As discussed above disparate studies have identified numerous HIV-1 *cis* elements in the gRNA (R, U5, PBS, ψ, and RRE) and viral *trans* factors (NC and MA of the Gag polyprotein, and Rev) that may act upon the gRNA at different steps of the encapsidation process, not to mention host cell proteins, which are not addressed by the work in this dissertation. What is not currently clear is if these viral components can function independently of each other, and more importantly, how the concerted actions of multiple viral components cedes efficient and specific encapsidation of RNA into HIV-1 viral particles. The primary focus of this dissertation investigates the proposal that the combination of viral *cis* elements and *trans* factors function in a common pathway to achieve RNA encapsidation. Identifying components of the encapsidation mechanism has primarily been accomplished through loss-of-function studies by mutational/genetic analysis. Since many viral *cis* elements and *trans* factors have pleiotropic effects, it can be cumbersome to isolate direct cause and effect relationships. Loss-of-function studies may also preclude the ability to elucidate how components may function together. A *bona fide* packaging system could be considered one that is transferrable to a heterologous RNA, resulting in a gain of

encapsidation function. The work presented in this dissertation describes how heterologous RNA systems were employed to investigate: i) the independent effect of the HIV-1 Rev/RRE system, as well as Rev orthologues, on heterologous RNA encapsidation into HIV-1 viral particles (Chapter 2); ii) the collaborative effect of the *cis* elements in the 5' UTR (R, U5, PBS, and ψ) and the Rev/RRE system on efficiency, and specificity, of heterologous RNA encapsidation (Chapter 3); and iii) the unique transduction properties of heterologous RNAs packaged into HIV-1 viral particles, and how they may prove advantageous for gene therapy protocols (Chapter 4). Lastly, conclusions to this work and future perspectives will be briefly discussed (Chapter 5).

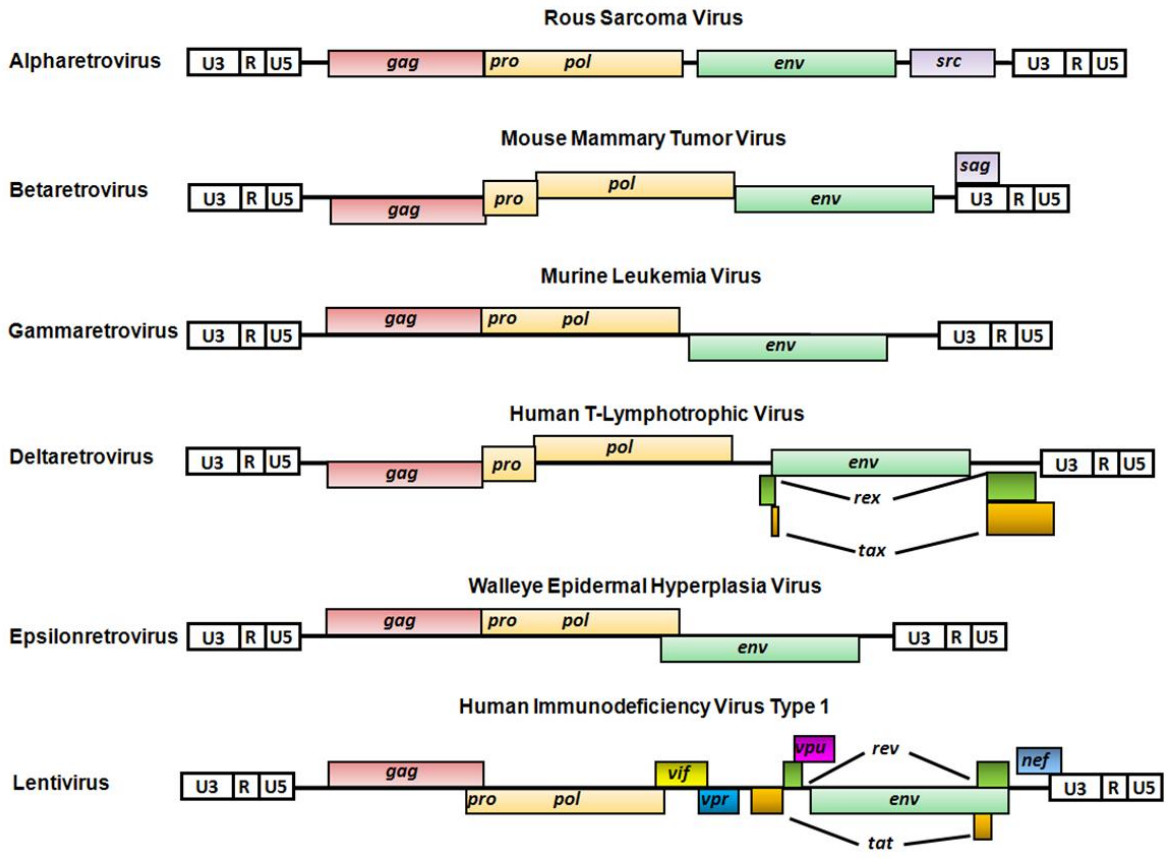


Figure 1. Retroviral Genome Organization. The proviral genome organization for a representative member of each genera of the orthoretrovirinae subfamily is depicted. Each genera contains two long terminal repeat (LTR) regions, a *gag* gene encoding structural proteins, a *pro* gene encoding protease, a *pol* gene that encodes reverse transcriptase and integrase, and an *env* gene encoding viral glycoprotein. The genera differ in the number of accessory proteins required for infection. Most complex are the lentiviruses with six accessory proteins two of which (Tat and Rev) are considered transactivators for viral replication.

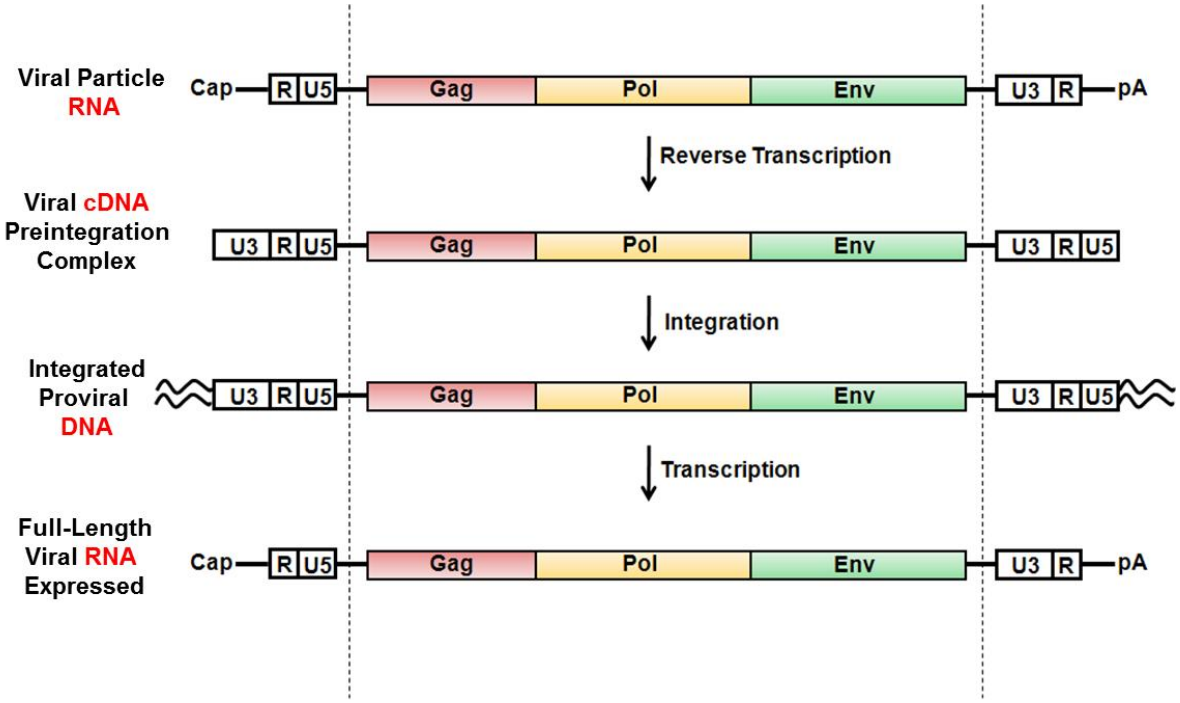


Figure 2. Retroviral Reverse Transcription Process. Viral RNA packaged into a viral particle is reverse transcribed by the viral reverse transcriptase into viral cDNA. The viral cDNA, in the context of a preintegration complex, enters the nucleus and viral integrase mediates integration into the host cell genome to generate a proviral DNA. The proviral DNA is then used as the template to express viral RNAs that generate viral proteins, as well as full-length genomic RNA that is packaged into nascent viral particles.

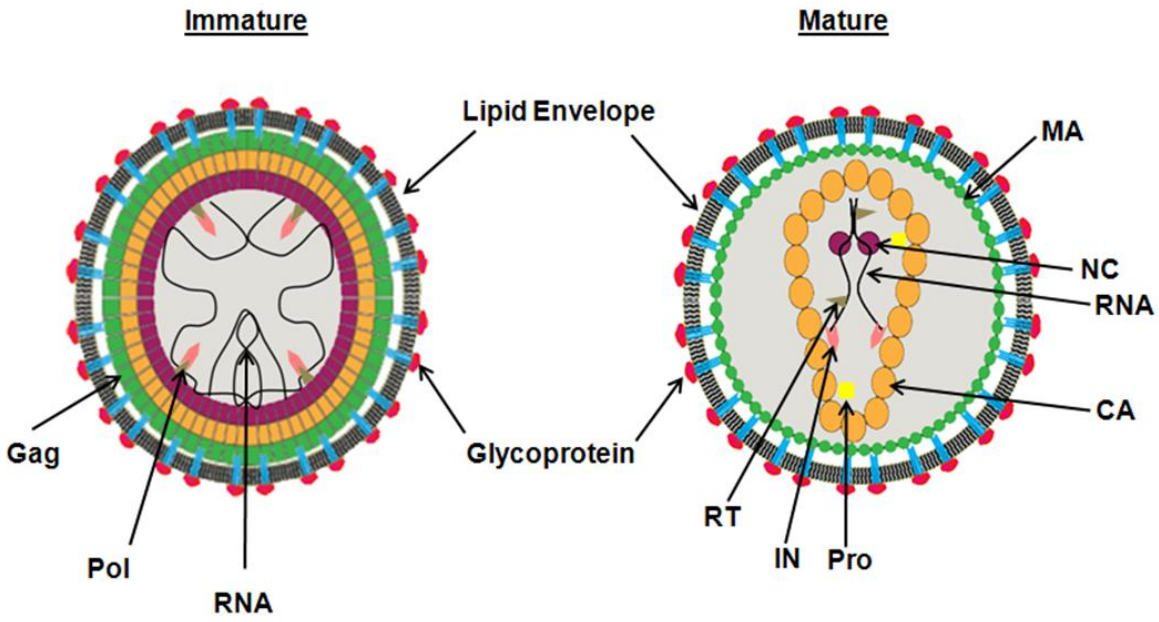
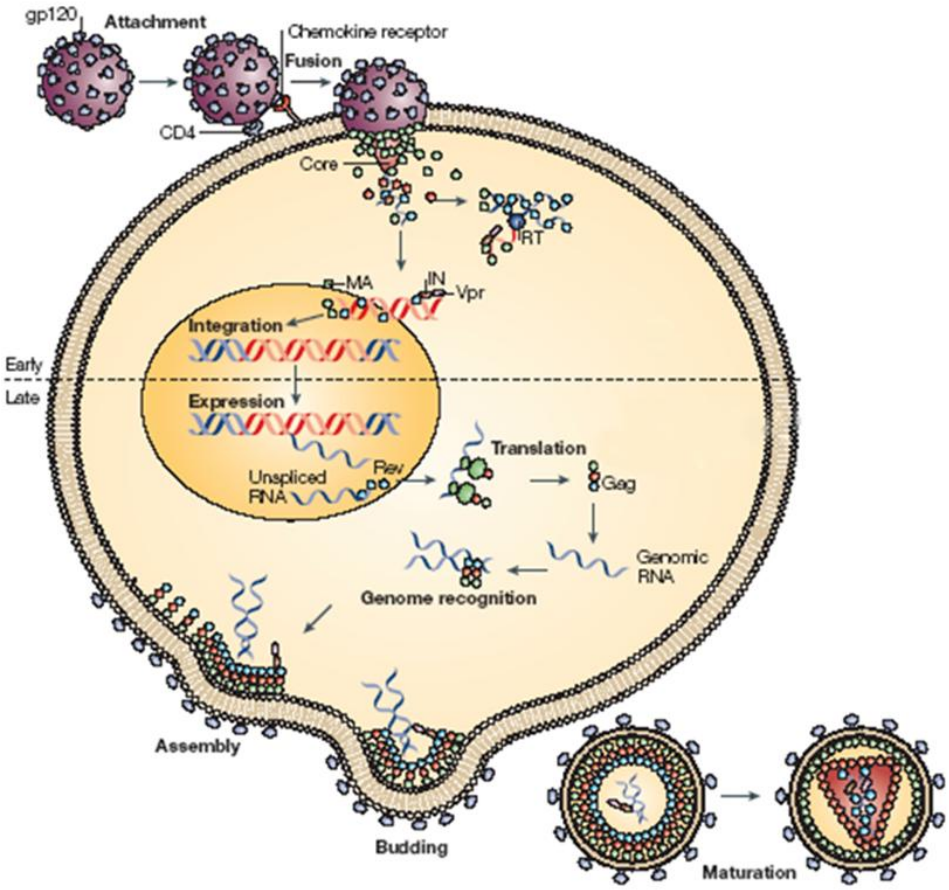


Figure. 3. Features of Immature and Mature Retroviral Particles. The features depicted are common to retroviruses. Nascent viral particles are initially formed as immature viral particles containing two molecules of ssRNA (black lines) packaged into a protein shell comprised of Gag and Gag-Pro-Pol molecules surrounded by a lipid envelope with viral glycoproteins embedded. The viral particle undergoes a readily observable morphological reorganization upon processing of the Gag and Gag-Pro-Pol Glycoproteins into constituent proteins to form the mature, infectious, viral particle.



Adapted by permission from McMillan Publishers LTD. D'Souza, V and Summers, MF. How Retroviruses Select Their Genomes. *Nature Reviews. Microbiology*. 2005. (3) 643-655.

Figure 4. Replication Cycle of HIV-1 Lentivirus. Early replication steps. A mature viral particle initiates infection by attaching to the host cell membrane through interaction of the viral envelope glycoprotein with a host cell receptor(s). The viral envelope fuses with the host cell plasma membrane, releasing the capsid and its contents into the cytoplasm. An uncoating of the viral capsid occurs, the RNA is reverse transcribed into viral cDNA, and enters the nucleus as a preintegration complex (MA [matrix protein], IN [integrase protein], and Vpr [accessory protein] are viral constituents of preintegration complex), whereby the viral cDNA can integrate into the host cell genome. **Late replication steps.** The proviral genomic DNA can be transcribed into viral RNAs that are full-length, partially spliced, or fully spliced RNAs. The full-length and partially spliced RNAs are exported from the nucleus through an interaction with the viral encoded accessory protein, Rev. Viral RNAs can be translated into structural and enzymatic proteins used to generate viral particles, and two molecules of full-length genomic RNA are encapsidated into nascent viral paticles assembling at the plasma membrane.

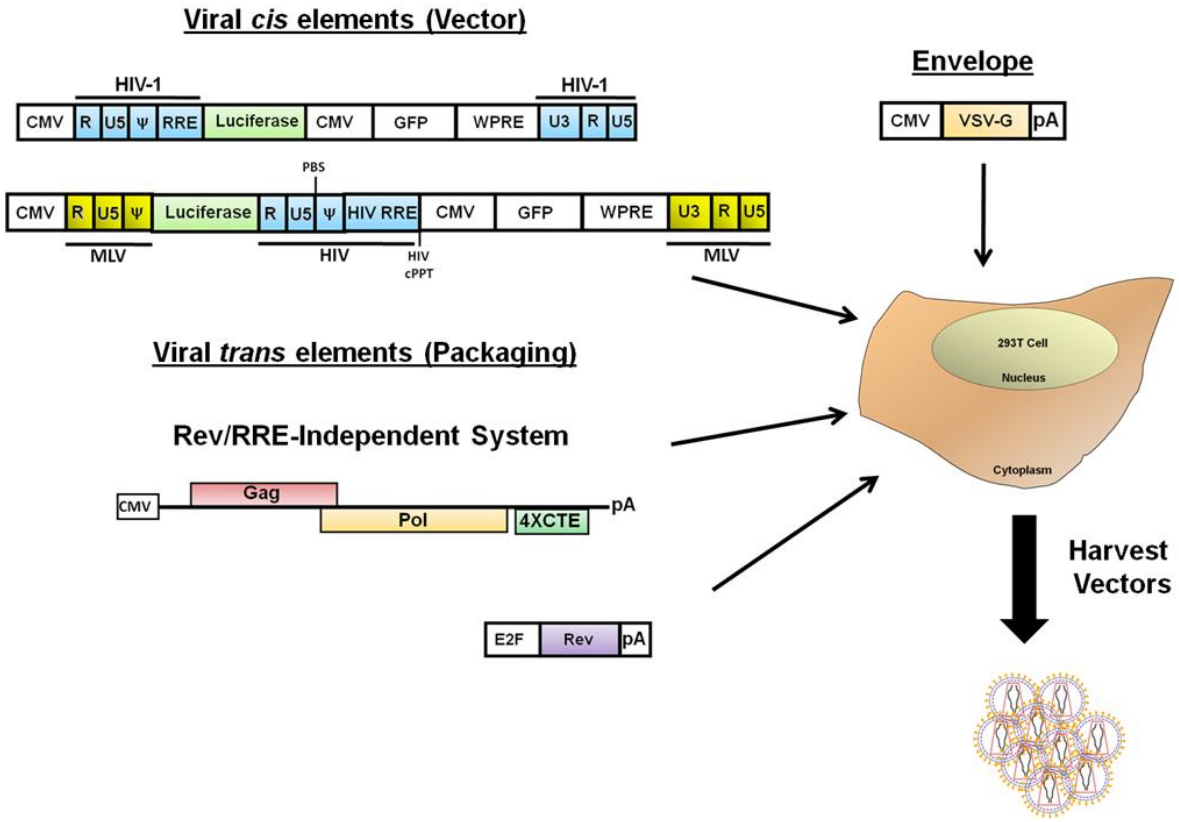


Figure 5. Retroviral Vector Production. For vector production the viral *cis* elements are separated from the viral *trans* elements onto individual plasmid constructs. ***Trans* Elements (Packaging Construct)**. The *trans* genes (*gag/pol*) encode the structural and enzymatic proteins that form nascent viral particles. In this case the packaging system depicted is a Rev/RRE-independent system, whereby viral particles can be generated in the absence of the Rev protein. This system is frequently utilized in experiments throughout the dissertation, allowing direct examination of the impact of Rev on encapsidation of the RNA into viral particles. ***Cis* Elements (Vector Construct)**. Viral *cis* elements can be readily manipulated in the context of the vector. The vectors depicted are a standard HIV-1 derived vector (top), and a MLV/HIV chimeric vector (bottom). The luciferase transgene, and CMV-GFP expression cassettes are used to monitor production and transduction, respectively. Vector particles are commonly pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) to confer vector particles with a broad host cell tropism. These constructs are cotransfected into 293T for vector production, and viral particles are harvested from the media.

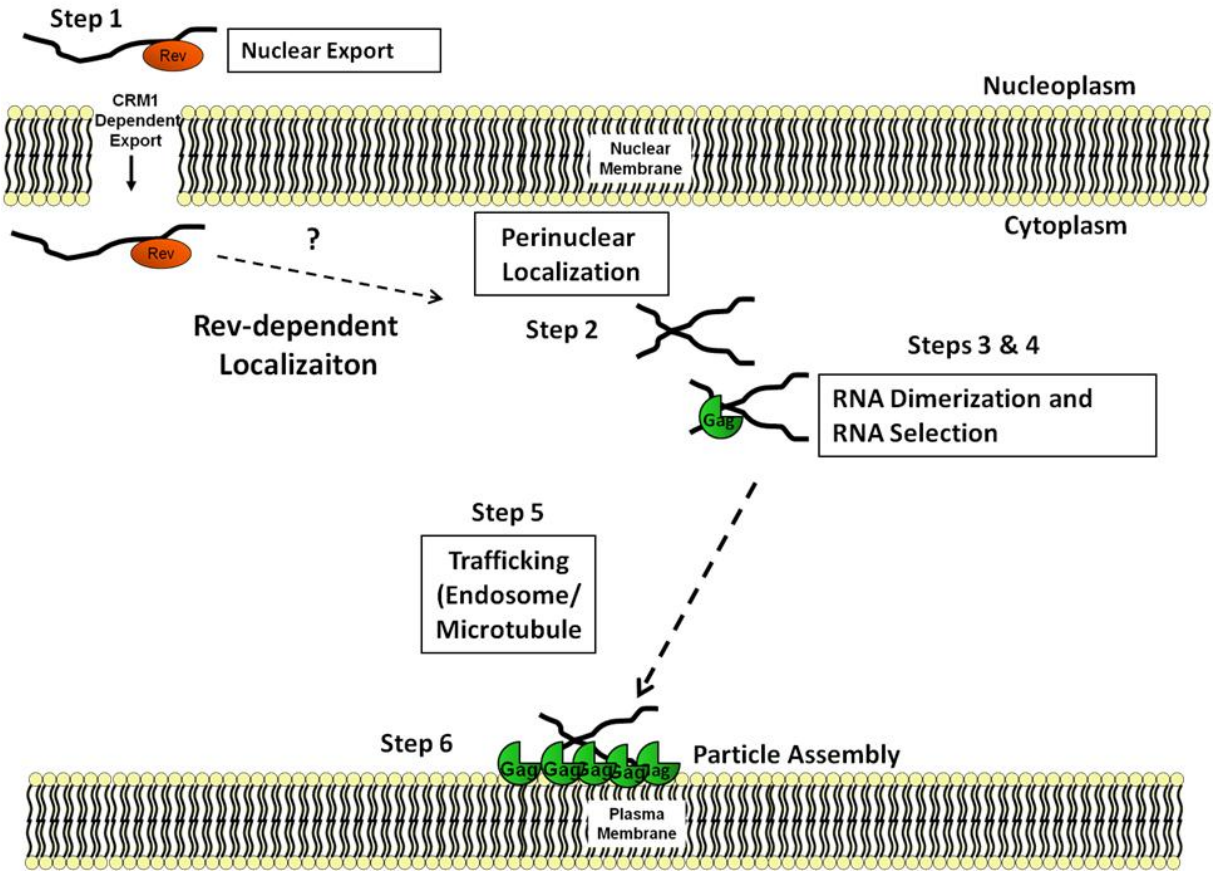
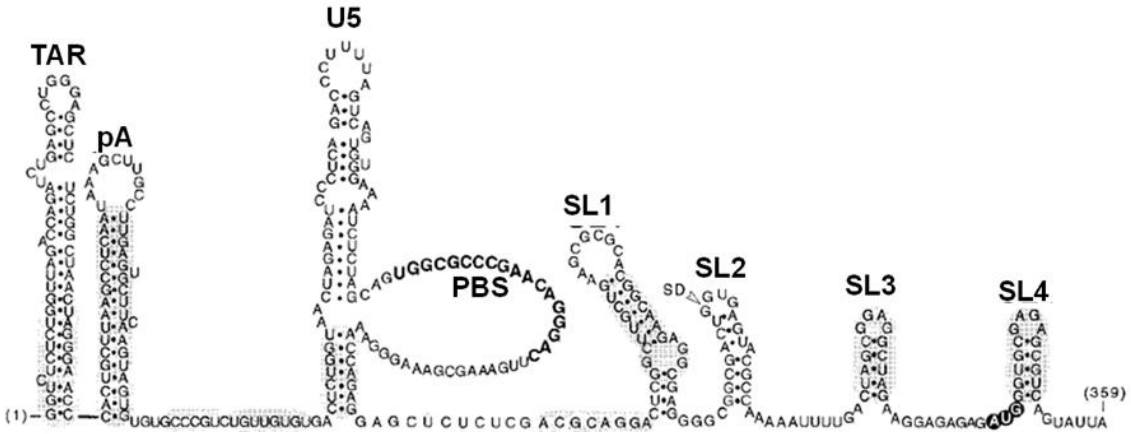
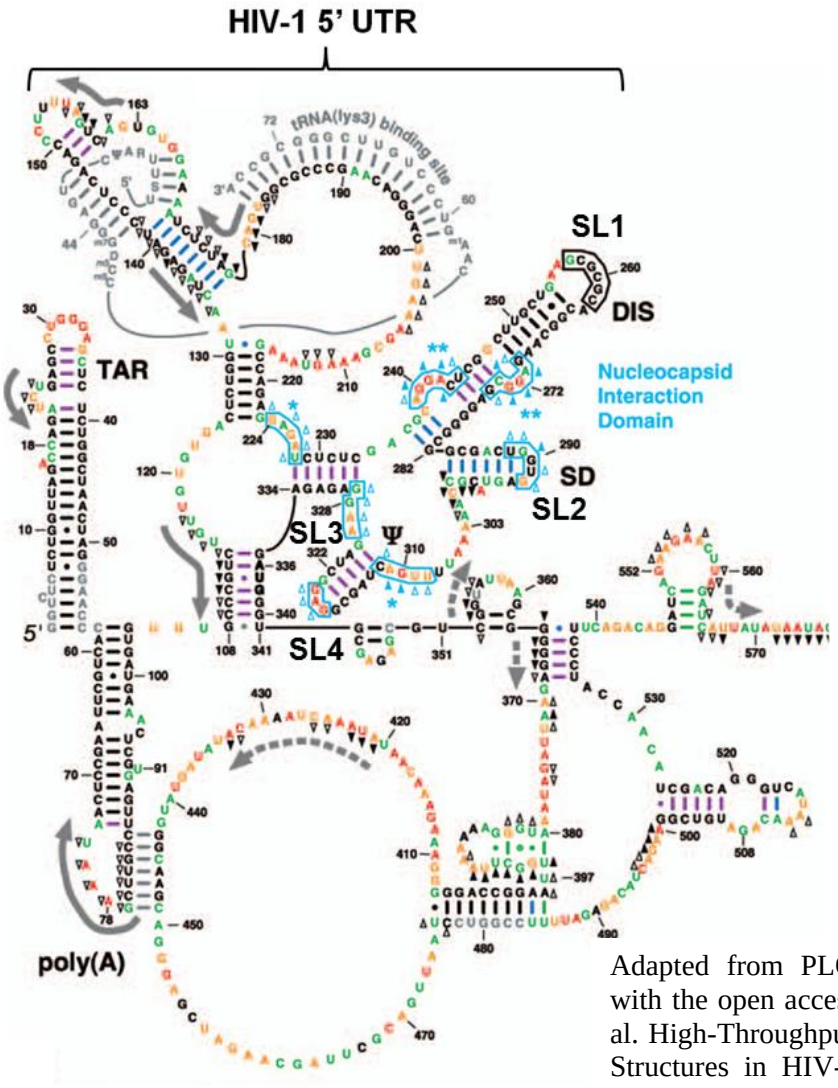


Figure 6. HIV-1 Genomic RNA Encapsidation Process. Step 1. Concomitant with transcription of full-length and partially spliced RNAs, the Rev protein interacts with its cognate Rev Response Element (RRE) to mediate nuclear export via the CRM1 dependent pathway. **Step 2**. Genomic RNA has been demonstrated to localize to perinuclear microtubule organizing centers. Work proposed in this dissertation indicates that Rev may make genomic RNA more amenable to subsequent Gag-RNA interactions, possibly through cytoplasmic localization (see chapter 3). **Step 3 & 4.** Dimerization of two molecules of genomic RNA occurs in the cytoplasm, and initial interaction of Gag with the canonical *cis* packaging signal are thought to occur in the cytoplasm prior to localization of the RNA to sites of particle assembly at the plasma membrane. **Step 5.** Trafficking of Gag-RNA complexes to the plasma membrane may occur via endosomes or microtubules. **Step 6.** Assembly of nascent viral particles occurs at the plasma membrane through numerous Gag-Gag interactions.



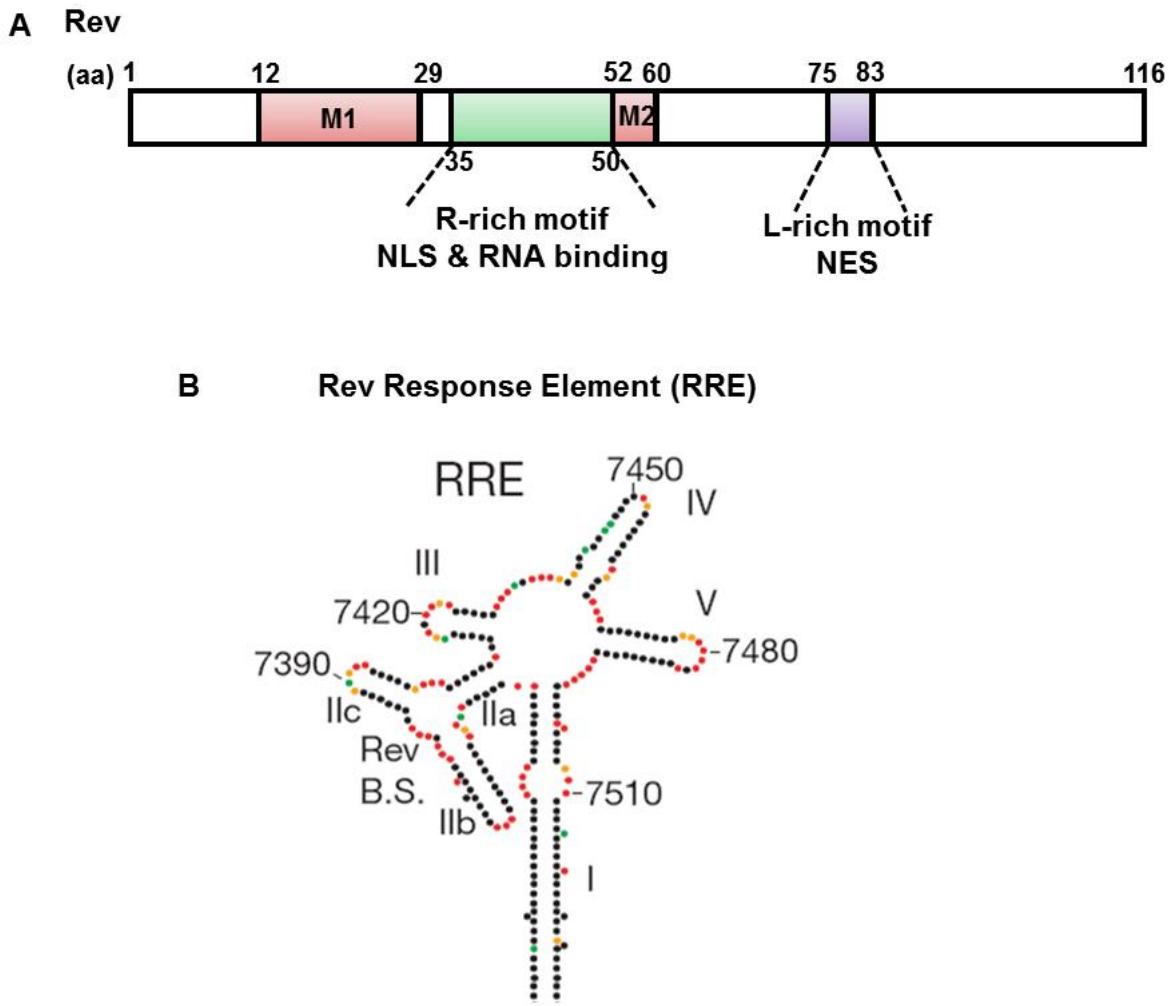
Adapted by Permission from American Society for Microbiology. Clever, J.L., Miranda Jr., D., & Parslow, T.G. RNA Structure and Packaging Signals in the 5' Leader Region of the Human Immunodeficiency Virus Genome. *Journal of Virology.* 2002. 76(23). 12381-12387.

Figure 7. RNA *Cis* Elements in the HIV-1 5' UTR. The 5' UTR is comprised of a series of stem-loops that impact nearly every aspect of the viral life cycle. The TAR (trans-activating response) element and polyadenylation signal are located in the R region of the 5' LTR. The U5 region harbors the *att* (attachment) sites at the 3' boarder with the PBS (primer binding site). *Cis* determinants within these regions have been shown to have dominant roles in viral RNA transcription, reverse transcription, & integration. However, several regions have also been implicated in encapsidation. Stem-loops SL1, SL2, SL3, and SL4 comprise what is considered the canonical packaging signal (ψ) and have all been demonstrated to be critical for encapsidation of genomic viral RNA. SL1 harbors the dimerization initiation site (DIS) in the loop; SL2 contains the major splice donor for HIV-1; SL2, SL3, and SL4 are thought to mediate high affinity interaction with the nucleocapsid protein; and SL4 is part of the 5' end that encodes the Gag polyprotein. Start site is indicated by AUG.



Adapted from PLOS Biology in accordance with the open access license. Wilkinson KA et al. High-Throughput SHAPE Analysis Reveals Structures in HIV-1 Genomic RNA Strongly Conserved Across Distinct Biological States. PLOS Biology. 2008. 6 (4) 883-899.

Figure 8. High Resolution SHAPE structure of the HIV-1 5' UTR. A high resolution RNA structure of about the first 570 nucleotides of HIV-1 are depicted. The strucuture elucidated by SHAPE revealed a highly complex structure with a number of long-range intramolecular interactions *in virio*. The TAR and polyadenylation (polyA) stem-loop structures in the R region are indicated. In gray the tRNA(lys3) is demonstrated as interacting with the PBS. Stem-loops 1, 2, 3, and 4 are indicated, where part of stem-loop 4 as shown in figure 7 is in a long range interaction with part of the U5 region. The dimerization initiation site (DIS) and major splice donor (SD) are indicated. Lastly, regions that the nucleocapsid interacts with are highlighted in blue. See reference for details regarding the SHAPE technology and nucleotide color coding scheme.



Adapted by permission from McMillan Publishers LTD. Watts JM et al. Architecture and secondary structure of an entire HIV-1 genome. *Nature*. 2009. 460. 711-719.

Figure 9. HIV-1 Rev and Rev Response Element (RRE). **A.** The Rev protein is comprised of multiple functional domains. Amino acids (aa) 12-29 and 52-60 constitute the two multimerization (M1 & M2) domains; aa 35-50 comprise the arginine rich motif (ARM) which governs nuclear/nucleolar localization signal (NLS) and overlaps with the RNA binding domain; and, aa 75-83 contain the leucine rich motif which constitutes the nuclear export signal (NES). **B.** The Rev Response element located in the RNA is a highly conserved element comprised of a series of stem-loop structures. The primary binding site for Rev is indicated by Rev B.S. and is primarily located in stem IIb. This RNA structure was obtained using the innovative SHAPE approach as described in the indicated manuscript.

Chapter 2

HIV-1 REV AUGMENTS ENCAPSIDATION OF HETEROLOGOUS RNAs INTO HIV-1 VIRAL PARTICLES[1]

Introduction

Encapsidation of HIV-1 gRNA is conventionally associated with the canonical *cis* packaging signal (ψ) located in the HIV-1 5' UTR of the gRNA, and extending into the 5' end of sequence encoding the *gag* gene. Transfer of this sequence is not sufficient to support encapsidation (15), therefore additional viral *cis* elements and *trans* factors have been investigated for effects on encapsidation. Encapsidation of RNA into nascent viral particles assembling at the plasma membrane requires the RNA to traverse the entire cell following initial transcription of full-length RNA in the nucleus. Viral-dependent mechanisms must ensure efficient and specific encapsidation of gRNA during the entire process of viral RNA translocation to sites of particle assembly. Interaction of the HIV-1 Rev protein with its cognate RRE in the gRNA, concurrent with transcription in the nucleus, make it a strong candidate for conferring both specificity and efficiency onto the HIV-1 gRNA early in the process of viral RNA encapsidation. Rev's ability to readily shuttle between the nucleus and cytoplasm allow it to influence viral RNA function in multiple capacities.

[1]A portion of the work in this chapter was published by Adam Cockrell, Henriette van Praag, Nicholas Santistevan, Hong Ma, and Tal Kafri, titled:The HIV-1 Rev/RRE system is required for HIV-1 5' UTR *cis* elements to augment encapsidation of heterologous RNA into HIV-1 viral particles (45).

The primary function ascribed to the Rev/RRE system is nuclear export of full-length, and partially spliced, HIV-1 RNAs through the CRM1 nuclear export pathway (176, 199). This is a highly conserved mechanism with orthologues for human and non-human primate lentiviruses including HIV-1, HIV-2, & SIV, as well as the non-primate feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), caprine arthritis-encephalitis virus (CAEV), visna virus (VV), Jembrana disease virus (JDV), and bovine immunodeficiency virus (BIV) lentiviruses (119, 130, 145, 164, 174). Even the more distantly related complex retroviruses human T lymphotropic virus type 1 (HTLV-1) and type 2 encode an HIV-1 orthologue, the Rex accessory protein, of which HTLV-1 Rex nuclear export domain was shown to be functionally interchangeable with HIV-1 Rev (86). Employing a viral encoded protein for nuclear export is unique to complex retroviruses. Simple retroviruses, such as MLV, accomplish nuclear export independent of viral encoded proteins (106, 193).

The nuclear export functions associated with the Rev/RRE have primarily been characterized using reporter systems (mainly chloramphenicol acetyltransferase [CAT], luciferase, or GFP), whereby successful translation of the reporter RNA is dependent upon the interaction of Rev with its cognate RRE in the RNA and subsequent nuclear export, as well as by measuring p24 capsid protein expression. Since these studies were predominantly designed to investigate Rev-dependent RNA nuclear export effects, additional downstream functions of Rev would have been difficult to detect. The conserved mechanism of Rev-dependent nuclear export through the CRM1 pathway compels the logic that the CRM1 pathway may dictate the fate of a viral RNA for downstream events of replication that occur in the cytoplasm. In line with this reasoning several reports demonstrate that the nuclear

export pathway may influence HIV-1 assembly (93, 94, 149, 191, 201). Protein expression assays facilitated the elucidation of Rev-dependent nuclear export through the CRM1 pathway; however, alternative measurements are necessary to distinguish nuclear export from additional Rev functions in the cytoplasm (68, 71).

In addition to nuclear export, an expanding body of data suggests that HIV-1 Rev can function in at least three additional capacities: i) enhancing translation of viral RNAs, ii) interfering with HIV-1 superinfections, and iii) influencing RNA encapsidation (68, 71). These functions are all downstream to nuclear export; therefore nuclear export is *a priori*. Distinguishing between Rev-dependent nuclear export and downstream events manifests the need to separate these functions. Studies have shown that Rev can enhance encapsidation efficiency by measuring RNA packaged into viral particles (directly and indirectly) relative to viral RNA available for packaging in the cytoplasm (5, 19, 67, 181). Previous studies have all examined the contribution of the Rev/RRE system to RNA encapsidation when the RRE is in the same RNA context as HIV-1 ψ, as well as other HIV-1 *cis* elements, therefore the Rev/RRE effect may be masked by the more dominant packaging components. Studies shown here were executed based on the proposal that isolating the Rev/RRE system from the influence of HIV-1 *cis* elements (i.e. canonical packaging signal) involved in encapsidation would demonstrate the independence of the Rev/RRE system to influence RNA encapsidation. Here, the Rev/RRE system was reconstructed in the context of heterologous RNAs to investigate the influence of the Rev/RRE system on RNA encapsidation into HIV-1 viral particles. The data demonstrate: i) for the first time that the HIV-1 Rev/RRE system can augment RNA encapsidation into HIV-1 viral particles in the absence of any canonical HIV-1 *cis* packaging signals; ii) HIV-1 Rev-mediated encapsidation is specific to heterologous

RNAs harboring the RRE; iii) FIV Rev (FRev) could function similarly to HIV-1 Rev (HRev) to enhance titer, and presumably RNA packaging, into HIV-1 particles, but EIAV Rev (ERev) could not; iv) that the HIV-1 RRE is not absolutely necessary for encapsidation, but it is essential to maintain an interaction between HIV-1 Rev and RNA; and v) that nuclear export through the CRM1 pathway is not absolutely necessary for Rev-dependent enhanced encapsidation.

Methods

Plasmid Constructs. Murine leukemia virus (MLV) and MLV/HIV chimeric vector constructs were derived from the MLV vector, pLNCX (146). The fundamental MLV vector as described in Fig. 10A was developed as follows: i) the WPRE (woodchuck hepatitis virus posttranscriptional regulatory element) was first subcloned into the *EcoRI/HindIII* sites of a pCLNCX self-inactivating (SIN) vector (154); ii) internal trans elements were replaced with a CMV-GFP cassette inserted by subcloning a *BamHI/XhoI* fragment from pTK113 (HIV-1 vector in Kafri lab), also retaining the HIV-1 cPPT-CTS (labeled cPPT in Fig. 1A) in this fragment; iii) the 3' SIN LTR was replaced with a complete MoMLV 3' LTR from pLNCX by subcloning the *HindIII/PmeI* fragment; and iv) the firefly luciferase (originally obtained from pBI-GL, Clontech) was subcloned into the *BamHI* site of the MLV vector generating pTK1328, the basic MLV vector in Fig. 10A. All notations of plasmids beginning with pTK are for ease of reference to the plasmid library in the Kafri lab. All subsequent MLV/HIV chimeric constructs were derived from pTK1328 by inserting various HIV-1 *cis* elements. All HIV-1 *cis* elements were derived from a standard HIV-1 vector from our lab, such as pTK113 (215). The MLV/HIV RRE vector (pTK1332) was derived from the basic MLV

vector by subcloning a *BamHI* fragment (~850 bp) comprising the RRE into a *BamHI* site of pTK1328.

The parental EIAV (UNC SIN 6.1W-1) and FIV (pFLX-CPL) vector constructs were kind gifts from the laboratories of Dr. John Olsen and Dr. Gary Nolan, respectively. The FIV/HIV RRE was generated in the following series of cloning: i) The WPRE was subcloned into the Cip treated *AleI* site of pFLX-CPL (pTK652); and ii) a large *NotI/SacII* fragment from pTK494 containing the HIV-1 RRE was subcloned into the *NotI/SacII* sites of pTK652 to generate pTK660. To generate the EIAV/HIV RRE chimeric construct a large *NotI/SacII* fragment containing the HIV-1 RRE, from pTK113, was subcloned into the *NotI/SacII* sites of UNC SIN 6.1W-1, yielding pTK728. Appropriate control constructs that contain the HIV-1 cPPT were generated as follows. The following cloning series was used for EIAV: i) a *BamHI/SacII* fragment from pTK113 was inserted into the *BamHI/SacII* sites of pBlueScript (pTK729); and ii) the *EcoRV/SacII* fragment from pTK729 was subcloned into UNC SIN 6.1 W-1 to generate the EIAV control (pTK730). For FIV control the following cloning series was executed: i) a PCR fragment containing the FIV cPPT from pCFWΔEnv packaging construct (kindly provided by Dr. Gary Nolan) was cloned into the *KpnI* site of pTK652; and ii) a *BamHI/XhoI* fragment containing the HIV-1 cPPT & CMV-GFP cassette was klenow treated and subcloned into the *EcoRV* site of pTK653 to yield the FIV control, pTK665.

The HIV-1 derived constructs were generated as follows. The standard HIV-1 vector with luciferase in the 5' end (pTK1363) was generated by subcloning a large fragment from a previously defined vector (pTK1305, described in chapter 3) into the *NotI/XhoI* sites of pTK1087 (a non-SIN HIV-1 vector with a complete U3 in the 3' LTR and internal CMV-GFP cassette). The HIV-1 ΔRRE vector (pTK1444) was derived from pTK1279 within

which the RRE was removed with *NotI/BamHI*. To generate pTK1444 the firefly luciferase gene was inserted into the *EcoRI* site of pTK1279. The HIV/9XMSRE ΔRRE construct (pTK1450) was derived from the following series: i) the MS2 9X response elements (pLMS29) were subcloned into the *BamHI/SpeI* sites of pBlueScript (pTK1448); ii) a *NotI/BglII* fragment containing the 9XMS2 response elements was then subcloned into *NotI/BamHI* sites of pTK1087 (pTK1449), while at the same time removing the HIV-1 RRE; and, iii) the firefly luciferase gene into the EcoRI site of pTK1449 to generate the HIV/9XMSRE ΔRRE construct, pTK1450.

The packaging constructs supplying necessary structural/enzymatic proteins were 4XCTE Gag-Pol (kindly provided by the laboratory of Dr. Christopher Baum), optimized Gag-Pol expression cassette (kindly provided by the laboratory of Dr. Donald Anson (60)), or ΔNRF (215). Expression of structural/enzymatic proteins from 4X CTE Gag-Pol, and optimized Gag-Pol is independent of all HIV-1 accessory genes, and encodes the complete *gag* and *pol* genes. The ΔNRF was described previously, but *gag-pol* gene expression is Rev-dependent.

The HIV-1 Rev protein was independently expressed from the EF-1α promoter in the E2F-Rev plasmid. The EIAV Rev expression cassette was a kind gift from the laboratory of Dr. John Olsen. The FIV Rev was initially obtained from the NIH AIDS reagent program and subcloned into *NcoI/XbaI* sites of pBlueScript (pTK1091). A CMV-FIV Rev expression cassette was then generated by subcloning a *EcoRI* fragment into pcDNA3.1zeo (pTK1095). The Rev-MS2 fusion was initially generated by PCR from a Rev-MS2 plasmid kindly provided by the laboratory of Dr. Thomas Hope. The Rev-MS2 PCR product was subcloned into the *HindIII/NotI* sites of pcDNA3.1zeo to generate pTK1163. In order to generate the

Rev-MS2 fusion the last amino acid of HIV-1 Rev was mutated from a glutamate to an aspartate (E116D), therefore a similar Rev positive control was generated by PCR to contain the E116D mutation. The PCR fragment was subcloned into the *HindIII/NotI* sites of pcDNA3.1zeo to generate the expression cassette, pTK1164. This construct was only used as a positive control in experiments where the Rev-MS2 fusion was employed. For experiments redirecting the nuclear export through the TAP/NXT1 pathway the following expression constructs were kindly provided by the laboratory of Dr. Marie-Louise Hammarskjold: RevM10-Tap fusion, RevM10 dominant negative mutant, NXT1, and TAP. The envelope protein was supplied from pMD.G, a VSV-G expressing construct.

Cells. 293T cells were maintained in DMEM (Hyclone) supplemented with 10% FBS (Invitrogen). Media was also supplemented with a 100X Antibiotic-Antimycotic solution containing penicillin, streptomycin, and amphotercin B (Cellgro).

Viral Particle Production and Concentration. Vector particles were produced by transient tranfection into 293T cells as described previously (44). Briefly, each 10cm dish of 293T cells was transfected with 15μg vector, 10μg packaging helper, 5μg VSV-G envelope, and 5μg Rev expressing plasmids. Experiments with Rev-MS2 constructs were performed with 10μg of each Rev plasmid. Plasmid amounts were compensated for in experiments in the absence of Rev with the empty plasmid construct, pCI-neo (Promega). Vector particles were harvested in conditioned media 48-60 hours post-transfection and filtered through a 0.45μm filter. Vector titers were determined by serial dilution on 293T cells and scoring for GFP positive cells using a Leica Leitz DMIRB inverted fluorescent microscope. Vector titers are expressed as values normalized to p24 (HIV-1 viral particles), or reverse transcriptase activity (MLV viral particles). The p24 assay and the reverse transcriptase assays are

described below. Concentration of vector particles was executed as done previously (44), with the exception that vector was purified over a single sucrose gradient, concentrated, and resuspended in 1X PBS.

HIV-1 p24 Capsid Concentration. Details of this assay were described previously (100). Briefly, EIA/RIA plates were coated with p24 antibody (NIH AIDS Research and Reference Reagent Program, #3537) at 1:1000 dilution and incubated overnight at 4°C. After blocking, samples/standards were treated with a 1% triton x-100 sample buffer, diluted appropriately, and added to plate for overnight incubation at 4°C. After washing, polyclonal rabbit anti-p24 antibody (NIH AIDS Research and Reference Reagent Program, #SP451T) at 1:300 was added to the plate, and incubated at 37°C for 3 hours. After washing, goat anti-rabbit IgG peroxidase (Pierce) at 1:15000 was added to the plate and incubated at 37°C for 2 hours. Assay was completed as described previously (100). Data used for titer normalization, and determining amount of viral particles for RNA isolation, are a mean of replicate samples.

FACS Analysis. Details of this assay were described previously (44). Briefly, at the indicated times post-transduction cells were fixed, and GFP expression was assessed on a Dako CyAn flow cytometer at the University of North Carolina Flow Cytometry Core Facility. Data was analyzed with Summit v4.3.01 software (Dako).

Luciferase Assays. Luciferase lysates were prepared by pelleting transfected 293T cells at the time of vector collection, and resuspending the pellet in 1X passive lysis solution (Promega). After freeze-thawing cell debris was pelleted by centrifugation for 15 minutes at 14,000 rpm and 4°C. Equivalent volumes of supernatant were assayed for firefly luciferase expression using 100 µl luciferin reagent (Promega). Luminescence was measured with a Victor^3 multilabel counter and Wallac 1420 Workstation software (Perkin-Elmer). Results

are expressed as relative light units (RLU)/mg protein. Protein concentrations were assayed according to manufacturer's instructions for Pierce BCA Protein Assay kit.

RNA Isolation. At 48-60 hours post-transfection vectors were harvested from the media, and cells were divided for luciferase assay, total protein, and cytoplasmic RNA/protein fractionation. Cells were removed from the plate by trypsinizing, and pelleted. The cytoplasmic fraction was separated by treating the pelleted cells with a MES (2-(*N*-morpholino)ethanesulfonic acid) buffered 0.1% Triton X-100 solution (10mM MES pH 6.5, 60mM KCl, 15mM NaCl, 5mM $MgCl_2$, 250mM sucrose, 0.1% Triton X-100) + protease inhibitor minicocktail (Roche) + 200 units/ml RNase Inhibitor (Fermentas). Pellets were incubated 2 minutes on ice, and nuclei were pelleted at 1500 rpm and 4°C. The cytoplasmic supernatant fraction was collected and separated for RNA isolation and protein analysis. Cytoplasmic fraction was monitored by western blot analysis for the absence of nucleolin protein, compared to total cellular proteins. RNA was purified from the cytoplasmic supernatant using the PARIS protein and RNA isolation kit (Ambion). Manufacturer's instructions were followed starting with addition of the 2X lysis/binding solution to an equal volume of cytoplasmic supernatant. Purified RNA was treated with DNase I Turbo (Ambion) for 1.5 hours at 37°C, and inactivated according to manufacturer's instructions. RNA integrity and concentration were regularly assessed by agarose gel electrophoresis. Cytoplasmic RNA was then utilized for analysis in qRT-PCR and northern blot.

Isolation of RNA from vector particles was carefully executed so that all vectors packaged into HIV-1 viral particles were normalized for equivalent amounts of p24 prior to isolation. For each sample at the time of RNA isolation p24 equivalents of vector particles and $5x10^6$ 293T cells were concomitantly added to the RLT lysis solution in preparation for

column purification of the RNA with the RNeasy Plus Mini kit (Qiagen). Spiking the vector particles with 293T cells at time of purification has multiple advantages: i) 293T cell RNA is a carrier during RNA purification; ii) RNA can be easily quantified after purification; and iii) quantity and integrity of purified RNA can be monitored by agarose gel electrophoresis. For purposes of homogenization the lysed particles/293T cell RNA were passed through a QIAshredder column (Qiagen), and subsequently through a genomic DNA eliminator column provided with the RNeasy plus mini kit. Lysate was then purified through the RNA isolation column provided with the RNeasy plus kit. Purified RNA was treated with DNase I Turbo (Ambion) as described for cytoplasmic RNA. Vector particle RNA was then utilized for analysis in qRT-PCR and northern blot. RNA was isolated from MLV viral particles in a similar fashion, except that viral particles were normalized for equivalent amounts of MLV reverse transcriptase. The reverse transcriptase assay is described above.

qRT-PCR. Purified cytoplasmic and vector RNA were heated to 65°C for 5 minutes prior to preparing each reverse transcription (RT) reaction. Each RT reaction was prepared at final concentrations of 1X RT Buffer (Qiagen OmniScript kit), 0.5mM each dNTP, 10 μM random prime nonamer, 10 units RNase Inhibitor (Fermentas), 500 ng RNA, and 4 units OmniScript reverse transcriptase (Qiagen). As controls for the presence of contaminating DNA each reaction was also performed in the absence of reverse transcriptase. Reactions were executed for 1 cycle on a BioRad MyCycler at 37°C for 1 hour. Equivalent amounts of cDNA were used in the subsequent quantitative PCR reaction. Each qPCR reaction was prepared at final concentrations of 1X ABI Taqman mix (2X Taqman Gene Expression Master Mix, Applied Biosystems), 0.9 μM forward primer, 0.9 μM reverse primer, and 0.1 μM probe. Reactions were performed at 1 cycle of 50°C/2 minutes, 1 cycle of 95°C/10 minutes, and 40 cycles of

95°C/15 seconds + 60°C/30 seconds on a 7300 real time PCR system (Applied Biosystems). All MLV and MLV/HIV chimeric vector RNAs were detected with a primer/probe set to the luciferase gene: For Luc 5'-aggtcttcccgacgatga-3', Rev Luc 5'-gtctttccgtgctccaaaac-3', and probe #70 (Roche Universal Probe Library). All HIV derived vector RNAs were detected with the following primer/probe set: HIV PS For 5'-aagcagggagctagaacgatt-3', HIV PS Rev 5'-cccagtatttgtctacagccttc-3', and probe #162 (Roche Universal Probe Library). All quantitative PCR reactions were normalized to an endogenous control reaction for TATA Binding Protein (TBP) using the following primer/probe set in independent reactions: For TBP 5'-gaaccacggcactgattttc-3', Rev TBP 5'-tgccagtctggactgttcttc-3', and probe #92 (Roche Universal Probe Library).

The levels of vector RNA (VRNA) and cytoplasmic RNA (CRNA) derived from the qRT-PCR data are expressed as arbitrary units (AU). The values were determined by normalizing the cycle threshold (C_t) for luciferase to that obtained for TBP, from each reaction. The calculation was as follows: $2^{-\Delta Ct}$, where the ΔC_t is (Luc C_t – TBP C_t). Independent reactions were performed for each VRNA and CRNA sample. All isolated VRNA could be normalized to TBP since each sample was copurified with 293T cell RNA, as described above. Calculation of the encapsidation efficiency, where VRNA relative to CRNA is expressed as AU, was determined for each independent sample. The calculation was as follow: $2^{-\Delta\Delta Ct}$, where the $\Delta\Delta C_t$ is (VRNA Luc C_t – VRNA TBP C_t) – (CRNA Luc C_t – CRNA TBP C_t). All relative encapsidation efficiencies are expressed as an average of at least three independent experiments with corresponding standard deviations (S.D.).

Northern Blot Analysis. Cytoplasmic RNA was isolated as described, and vector RNA was isolated from concentrated vector particles. Vector RNA was prepared from equivalent levels

of p24, and at the time of isolation the samples were copurified with 293T cells as described above. Prior to resolving RNA on a denaturing formaldehyde agarose gel, RNA was denatured at 65°C for 10 minutes. Equivalent amounts of cytoplasmic RNA, and vector RNAs, were resolved on the gel. Northern blot analysis was performed under standard conditions. Probes were random prime labeled with α^{32}P-dCTP (Easy Tide Deoxycytidine 5'-triphosphate, Perkin-Elmer) at 37°C for 1 hour. The probe was a fragment generated to the GFP at the 3' end of the vector RNA. Images were obtained on BioMax MR film (Kodak), or by phosphorimager (Molecular Dynamics Storm System).

Western Blot Analysis. Cytoplasmic and total cell lysates were resolved by standard denaturing SDS-PAGE analysis on 10% gels and blotted to Hybond P membrane. Membranes were probed with rabbit anti-nucleolin (1ug/ml; Abcam, cat# ab22758-100) and rabbit anti-GAPDH (1:500; Santa Cruz Biotechnology, cat# FL-335). Detection was achieved by probing blots with goat anti-rabbit IgG peroxidase (1:40000; Pierce). Rev and Rev-MS2 fusion proteins were examined with mouse anti-Rev (1:200; Santa Cruz Biotechnology, cat# sc69729). Detection was achieved with a goat anti-mouse IgG peroxidase (1:500). All antibody incubations and washes were done in 1X PBST. Signals were detected using ECL (GE Healthcare).

Results

The HIV-1 Rev/RRE augments encapsidation of heterologous RNAs in the absence of canonical HIV-1 *cis* packaging elements. The Rev/RRE system was recently demonstrated to enhance encapsidation of a HIV-1 vector RNA into HIV-1 viral particles (19). In this previous work, however, the Rev/RRE system was examined in the context of HIV-1 vectors

that contain *cis* elements from the 5' UTR including R, U5, PBS, and ψ regions that may also have contributed to the enhanced encapsidation. Additionally, the combination of the Rev/RRE system and *cis* elements from the 5' UTR may have pleiotropic effects which impact other stages of the viral life cycle (i.e. reverse transcription (1)). To isolate encapsidation effects directly attributable to the Rev/RRE system we assembled a heterologous RNA system derived from murine leukemia virus (MLV) vector RNA (Fig. 1A). Nuclear export of MLV vector RNA is autonomous (106, 193), a feature exploited to elucidate the effects of the Rev/RRE on HIV-1 processes (i.e. encapsidation). Heterologous vector RNAs were packaged into viral particles generated from a helper system (Gag/Pol-4X CTE) that does not rely upon the Rev/RRE system for nuclear export of RNA encoding the structural and enzymatic proteins (Fig. 10A). By circumventing dependence upon Rev for nuclear export of *gag/pol* RNA, and vector RNA, we could directly analyze the effect of Rev on encapsidation. The MLV/HIV RRE chimeric vector was constructed to express enhanced green fluorescent protein (eGFP) from an internal CMV promoter to provide an indirect indication of the RNA incorporated into viral particles (Fig. 10A). Vector titers were determined by scoring for GFP expression following transduction of 293T cells, and normalized to amounts of p24 capsid protein (Fig. 10B). Incorporation of only the HIV-1 RRE into the heterologous MLV vector (MLV/HIV RRE vector) enhanced titers 11-fold in the presence of Rev. This is the first report that the Rev/RRE system can influence packaging of a foreign RNA into HIV-1 viral particles. These results indicate that the HIV-1 Rev/RRE system can impact titers of vectors devoid of HIV-1 *cis* elements known to affect RNA packaging. Furthermore, the Rev/RRE system conferred corresponding increases in transduction of 293T cells exposed to equivalent amounts of p24 capsid protein (Fig. 11A

and B), demonstrating that the Rev mediated increase in titer cannot be attributed to Rev effects on HIV-1 particle production. Since it is well established that the Rev protein enhances nuclear export of RNAs containing a RRE, observed increases in titers may be a consequence of augmented nuclear export. This possibility was investigated by measuring the cytoplasmic vector levels in the producer cells.

The vectors were configured to indirectly assess cytoplasmic levels of vector length RNAs during production by situating the firefly luciferase gene such that it was included in the full-length vector RNA, but not in RNAs expressed from the internal promoter (Fig. 10A). Luciferase expression in the 293T producer cells is indirect evidence for cytoplasmic RNA, and may be subject to translational influences. Luciferase levels were marginally affected by Rev, indicating that Rev did not influence nuclear export of the MLV/HIV RRE vector (Fig. 10C). Examining the titer/luciferase ratio (Fig. 10D) revealed that the effects of the Rev/RRE system primarily alter vector titers (14 fold) with minimal cytoplasmic changes. Reasoning that the Rev/RRE system may mediate packaging of vector RNA into HIV-1 viral particles we were encouraged to further explore the mechanism mediating RNA encapsidation.

Encapsidation efficiency is a measure of RNA packaged into viral particles relative to the RNA available for packaging, in the cytoplasm. Vector RNA was isolated from viral particles in the media of 293T producer cells and vector producing cells were fractionated to obtain cytoplasmic RNA. Cytoplasmic separation was routinely monitored using western blot analysis for the absence of the nuclear specific protein, nucleolin, from the cytoplasm (Fig. 12A). Additionally, total, cytoplasmic, and viral RNA was regularly monitored for RNA integrity and to ensure equivalent loading levels by resolving on formaldehyde denaturing

agarose gels (Fig 12B). In the process of isolating viral RNA samples are copurified with cellular RNA to serve as controls for RNA integrity, and amounts. Relative RNA levels in viral particles and cytoplasm of producer cells were examined by qRT-PCR and northern blot analysis (Fig. 13). In the absence of all HIV-1 5' UTR *cis* elements Rev enhanced the levels of MLV/HIV RRE vector RNA in viral particles 6 fold compared to the MLV vector (Fig. 13A), which is in line with what was observed for the titers (Fig. 10B). Compared to the basic MLV vector the Rev/RRE system did not impact cytoplasmic vector RNA levels (Fig. 13B), thus the increase in RNA encapsidation is similar to that observed in viral particles, 7-fold (Fig. 13C). Northern blot analysis supported the observation that the Rev/RRE system could augment encapsidation independent of additional HIV-1 5' UTR *cis* elements (Fig. 13D). Specificity is also apparent from northern blot analysis, demonstrating that vector length RNA species is more efficiently packaged in the presence of Rev than a smaller RNA species (labeled as GFP generated from the internal promoter) lacking the RRE *cis* element. The HIV-1 Rev/RRE system can confer an encapsidation advantage onto a heterologous MLV vector RNA.

The capacity of the HIV-1 Rev/RRE system to enhance encapsidation, and confer specificity, upon a heterologous RNA is not restricted to the MLV vector system. The HIV-1 RRE was also incorporated into non-primate lentiviral vector RNAs, derived from feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV), to examine RNA encapsidation into HIV-1 viral particles (Fig. 14A). HIV-1 viral particles were derived from the Rev/RRE-dependent ΔNRF packaging construct, which supplies Rev *in trans* (Fig. 14A). Incorporation of the RRE enhanced vector titers for both EIAV (14 fold), and FIV (18 fold), an indication that the Rev/RRE system may increase heterologous RNA encapsidation into

HIV-1 viral particles (Fig. 14B). These results were substantiated by northern blot analysis demonstrating significant Rev/RRE-dependent increases of EIAV, and FIV, vector RNAs into HIV-1 viral particles (Fig. 14C). Similar to the chimeric MLV/HIV RRE vector in the presence of Rev the smaller GFP species, lacking RRE, was nearly undetectable, even though an abundance of this RNA species could be detected in the cytoplasm. These results provide further evidence that the HIV-1 Rev/RRE system can confer specificity onto a heterologous RNA for encapsidation. Using the ratio of vector titers relative to the cytoplasmic RNA (northern blot) available for packaging, an indirect measure of the encapsidation efficiency showed that the HIV-1 Rev/RRE system could dramatically augment encapsidation of EIAV (13 fold) and FIV (16 fold) vector RNAs into HIV-1 viral particles (Fig. 14D). Combined with previous data, this work demonstrates that the encapsidation effect of the HIV-1 Rev/RRE system is not restricted to a single RNA species, but rather heterologous transfer of the RRE to at least three different RNA species confers increased RNA packaging efficiency into HIV-1 viral particles.

Importantly, this work also indicates that the HIV-1 Rev/RRE encapsidation function may be conserved among lentiviruses. Homologous Rev proteins derived from distantly related non-primate lentiviruses, EIAV (ERev) and FIV (FRev), revealed distinct titers, which are presumably related to RNA encapsidation effects (Fig. 15). FRev dramatically augmented titers of FIV vector RNAs containing the FIV RRE (83- & 134-fold) (Fig 15A). Since the FRev is augmenting titers of FIV vectors into HIV-1 viral particles, the data suggest that FRev may utilize an encapsidation mechanism also shared by HIV-1 Rev (HRev). In contrast ERev did not enhance titers of EIAV vector RNAs containing EIAV RRE, into HIV-1 derived viral particles (Fig. 15B). In both cases the HRev augmented

encapsidation of RNAs containing the HIV-1 RRE (Fig 15A & B). Apparently, ERev may utilize an encapsidation mechanism distinct from that of HRev and FRev.

Overall, the data demonstrate that the HIV-1 Rev/RRE system can enhance encapsidation of heterologous RNAs into HIV-1 viral particles independent of the canonical HIV-1 packaging signal, as well as other 5' UTR *cis* elements. Furthermore, HRev and FRev may utilize a common encapsidation mechanism, whereas ERev may mediate encapsidation in a unique manner. These data indicate that the Rev/RRE system may be a central component of a RNA encapsidation mechanism that is traditionally associated with *cis* elements in the 5' UTR (R, U5, PBS, and ψ). Since the Rev/RRE system is central to encapsidation part of the mechanism to achieve specificity may comprise an independent function associated with the RRE and nuclear export through the CRM1 pathway.

The HIV-1 Rev-enhanced encapsidation effect is dependent upon maintaining an interaction with the RNA. The abovementioned results demonstrated that HIV-1 Rev could augment encapsidation of heterologous RNAs upon transfer of the RRE *cis* element to a heterologous RNA. These data imply that Rev may interact with the RRE *cis* element to mediate encapsidation into HIV-1 viral particles. In addition, the HIV-1 RRE *cis* element may have a functional role in the encapsidation mechanism beyond maintaining an interaction between the Rev protein and the RNA to be encapsidated. This possibility was further investigated by initially deleting the RRE *cis* element from a typical HIV-1 vector RNA (Fig. 16A), and measuring vector RNA encapsidation in the presence and absence of HIV-1 Rev (Fig 16). Vector titers indicated that deletion of the RRE may have prevented RNA encapsidation (Fig. 16B), which was bolstered by measuring the titer-to-luciferase ratio

(Fig. 16C). In two independent experiments measuring encapsidation efficiency by qRT-PCR demonstrated that deletion of the RRE obviated the Rev-mediated encapsidation effect, compared to control HIV-1 vector RNA containing the HIV-1 RRE (Fig. 16D). These data insinuate that the Rev-mediated encapsidation effect is dependent upon the HIV-1 RRE. However, it is still a possibility that the RRE serves a role in the encapsidation mechanism. A better understanding can be gleaned by substituting the RRE with an alternative *cis* RNA binding element.

The bacteriophage MS2 system was previously employed to study the effects of Rev mutants on nuclear/nucleolar import and nuclear export (142). Along similar lines the HIV-1 RRE was replaced with nine tandem copies of high affinity MS2 response elements in a standard HIV-1 vector RNA to generate HIV/9X MS2RE ΔRRE vector (Fig. 17A). Concomitantly, a fusion of the bacteriophage MS2 coat protein with the HIV-1 Rev protein was ectopically expressed during vector production in 293T cells to examine the effects on RNA encapsidation. Western blot analysis demonstrated overexpression of the Rev-MS2 protein (Fig. 17B). The Rev-MS2 fusion was comparably functional to the Rev protein as demonstrated by standard HIV-1 vector titers (Fig. 17C), and titers normalized to luciferase (Fig. 17D). However, only the Rev-MS2 fusion protein augmented the packaging of HIV/9X MS2RE ΔRRE vector RNA into HIV-1 viral particles (7 fold), as measured by titer/luciferase ratio (Fig. 17D). These data indicate that the HIV-1 RRE is not necessary for enhanced encapsidation, but rather that an interaction should be maintained between the HIV-1 Rev protein and the RNA. However, RNA encapsidation with the Rev-MS2 system is not as efficient as the Rev/RRE system, which may suggest that the RRE is most desirable as the cognate Rev *cis* element; or, that it may be technically challenging to reproduce the Rev/RRE

interaction with a synthetic system. Further discussion of these concepts is shown below. Since Rev is important for efficient and specific encapsidation of RNA available in the cytoplasm; conceivably, the fate of RNA to be encapsidated in the cytoplasm may be determined by the nuclear export pathway.

Rev-enhanced encapsidation does not require nuclear export through the CRM1 nuclear export pathway. A number of reports have demonstrated that the nuclear export pathway can influence retroviral particle assembly (93, 94, 149, 191, 201). Presumably, the nuclear export pathway may also be part of a mechanism that determines the fate of RNAs in the cytoplasm. Nuclear export of full-length and partially spliced viral RNAs through the CRM1 nuclear export pathway is the primary post-transcriptional regulatory mechanism ascribed to the Rev/RRE system; therefore, the CRM1 pathway may also be central to efficient RNA encapsidation. To examine this possibility HIV-1 vector RNAs were redirected from the CRM1 pathway to nuclear export through the Tap/NXT1-dependent pathway (Fig. 18A). The TAP/NXT1 pathway is the most common pathway utilized for nuclear export of spliced cellular mRNAs. A dominant negative Rev mutant (RevM10) contains a mutation in the nuclear export signal that prevents an interaction with CRM1 (131); nonetheless, retains the ability to interact with the RNA. A RevM10-Tap fusion was employed to redirect RNA from the CRM1 pathway to the Tap/NXT1 pathway (Fig. 18A). This fusion was previously demonstrated to facilitate nuclear export in a CRM1-independent manner (74). The system was tested using a pMDL HIV-1 packaging construct whereby p24 expression is Rev/RRE-dependent (Fig. 18B). Ectopic expression with the RevM10-Tap fusion and the NXT1 cofactor demonstrated high levels of viral particle production as

measured by p24 capsid protein in the media (Fig 18C), whereas the RevM10 mutant alone did not promote p24 expression. Therefore, the RevM10-Tap/NXT1 system mediates nuclear export in a CRM1-independent manner.

The nuclear export effect on encapsidation was investigated by ectopically expressing the RevM10-Tap/NXT1 system, and testing the capacity to encapsidate a standard HIV-1 vector RNA with an RRE (Fig. 18A) into HIV-1 viral particles derived from a codon optimized HIV-1 Gag-Pol packaging system. This packaging system affords Rev-independent and Tap-independent production of viral particles. The RevM10-Tap/NXT1 system augmented vector titers 6 fold (Fig. 19A), indicating that there may be enhanced packaging of vector RNA. Cytoplasmic RNA levels may not be influenced by the RevM10-Tap/NXT1 system since the titer/luciferase ratio (5 fold increase, Fig 19B) indirectly indicated a similar increase in packaging. These data were confirmed by qRT-PCR demonstrating that the RevM10-Tap/NXT1 system mediated a 7 fold increase in RNA encapsidation efficiency (Fig. 19C). Together, these two methods indicate that the Rev-mediated encapsidation effect is not strictly dependent upon CRM1 nuclear export. However, the efficiency of the system does not achieve levels similar to that of wild-type HIV-1 Rev through the CRM1 pathway. Therefore, although not absolutely required for the Rev-enhanced encapsidation effect, nuclear export through the CRM1 pathway is optimal for efficient RNA encapsidation.

Discussion

The objective of these studies was to understand the role of the HIV-1 Rev/RRE system in the sophisticated mechanism of selecting a RNA species from the milieu of host cell RNAs

to ensure efficient and specific encapsidation into nascent HIV-1 viral particles. Isolating the Rev/RRE system away from other HIV-1 *cis* elements demonstrated for the first time that the Rev/RRE system can augment encapsidation of heterologous RNAs into HIV-1 viral particles (Figs. 10-14). Importantly, encapsidation was achieved in the absence of more conventional HIV-1 *cis* packaging elements located in the 5' UTR of the full-length HIV-1 RNA. Nevertheless, the contribution of HIV-1 *cis* elements in the 5' UTR to encapsidation should not be marginalized, and is addressed in Chapter 3 of this dissertation. Prior studies investigated a role for HIV-1 Rev in the encapsidation process in the presence of the major *cis* packaging elements in the 5' UTR (5, 19, 67, 181); however the Rev effect could have been compounded by pleiotropic packaging effects of additional *cis* elements from the HIV-1 5' UTR. The capacity of the HIV-1 Rev/RRE to enhance encapsidation in the absence of other HIV-1 *cis* elements suggests that the interaction of the Rev protein with the RRE can make the RNA amenable to specific encapsidation into HIV-1 viral particles, and that the Rev/RRE system may impact encapsidation at steps preceding the involvement of *cis* elements in the 5' UTR.

A number of scenario's may help explain the mechanism by which Rev functions in RNA encapsidation. A plethora of cellular proteins have been demonstrated to function as Rev/RRE cofactors, possibly as ribonucleoprotein complexes, that can influence nuclear import, splicing, nuclear export, RNA release, and subcellular localization, ultimately impacting viral replication in a positive or negative manner (41, 71, 199). Rev may localize the viral RNA in the cell cytoplasm, thereby facilitating efficient/specific encapsidation. Several cellular proteins have been shown to alter the location of Rev (47, 56, 80, 147, 180, 189, 196, 222, 223). Recently the host cell double-strand RNA binding protein, NF90, was

demonstrated to alter the subcellular location of Rev and influence HIV-1 viral particle production (206); implying that the localization of viral RNA may also be altered. On the other hand, the HIV-1 Rev/RRE system may recruit host cell proteins that manipulate RNA structure or promote chemical modifications to the viral RNA that effect cytoplasmic processes such as translation and/or encapsidation. The host cell nuclear protein Matrin 3 was demonstrated to interact with RRE-containing RNAs (115, 220) and influence post-transcriptional viral gene expression in a Rev/RRE-dependent fashion (220). Additionally, a recent report demonstrated that Rev can recruit the cellular methyltransferase protein, PIMT, which trimethylates the 5' RNA cap of some HIV-1 RNAs as a regulatory mechanism for selective expression (221). Nevertheless, there is a dearth of evidence implicating host cell proteins in HIV-1 RNA packaging. The only host protein implicated in Rev/RRE-dependent packaging of HIV-1 viral RNA has been the RNA helicase protein DDX24, whereby down-regulation of DDX24 was demonstrated to decrease the amount of HIV-1 RNA in viral particles (126). Most likely a Rev/RRE-dependent encapsidation mechanism should involve recruitment of cellular proteins, probably in the context of a ribonucleoprotein complex (41).

A Rev/RRE-mediated RNA encapsidation mechanism may also be conserved among primate and some non-primate lentiviral Rev proteins. Data presented here suggest that FIV Rev may function similar to HIV-1 Rev (Fig. 15). Along these same lines previous data demonstrated that SIV Rev can also augment encapsidation of SIV RNA into SIV viral particles (19). Additionally, like HIV-1 Rev, both FIV and EIAV Rev proteins utilize a CRM1-dependent nuclear export mechanism (164). In contrast, EIAV Rev did not influence encapsidation of standard EIAV or EIAV/HIV chimeric vector RNAs into HIV-1 viral particles (Fig. 15B). This is despite the fact that HIV-1 Rev could enhance titers of

EIAV/HIV chimeric vectors (Figs. 14 & 15), and encapsidation of chimeric vector RNA into HIV-1 viral particles (Fig. 14). The unique properties of HRev, FRev, and ERev may be exploited to identify unique host cell factors that are common to HRev and FRev, but not ERev, thereby providing further insight into the RNA encapsidation process.

Rev can clearly influence the utilization of viral RNAs for different late viral replication processes (i.e. translation & packaging) through interaction with the RRE. However, it is not clear if the RRE is absolutely necessary for Rev to mediate these late replication processes. Deletion of the RRE eliminated encapsidation of HIV-1 vector RNA (Fig. 16). Replacing the RRE with a heterologous RNA binding element (i.e. MS2 bacteriophage coat protein response elements inserted in tandem into HIV-1 vector RNA) was sufficient to augment titers and encapsidation as indirectly measured by titer/luciferase (Fig. 17). These results are in agreement with previously published reports demonstrating that heterologous RNA binding elements are sufficient to support RNA packaging if the interaction between Rev and the vector RNA is retained (125). Nonetheless, the efficiency of encapsidation does not achieve levels comparable to those of the Rev/RRE system, as demonstrated here (Fig. 17), and in previous work (19). Although this may indicate that the RRE is important to maintain an efficient level of RNA encapsidation, technical differences in the systems may also explain the distinction. A central aspect of Rev function is the capacity to oligomerize on the RRE (52, 133, 177, 225). In the context of the Rev-MS2 protein, Rev may be restricted to only forming dimers since each MS2 response element can recruit only two MS2 molecules (105), thereby possibly limiting Rev function. Notably, however, the Rev-MS2 fusion retained function similar to the Rev protein alone, yielding comparable titers of an HIV-1 vector with the RRE (Fig. 17). An alternative possibility is

that the Rev-MS2 fusion protein does not interact efficiently with the vector RNA to mediate subsequent encapsidation. The Rev protein is efficiently imported into the nucleus where it interacts with RRE-containing viral RNA. If the RRE is necessary for proper localization of viral RNA to sites amenable to interaction with the Rev protein, then replacing the RRE with the MS2 response elements may diminish the potential for this interaction to occur, thereby negatively influencing downstream events. In this case the RRE would be important for efficient RNA encapsidation.

The Rev/RRE interaction governs nuclear export of full-length and partially spliced viral RNAs (i.e. those containing a HIV-1 RRE *cis* element) through the CRM1-dependent pathway. This is in contrast to the preponderance of spliced cellular RNAs which utilize the Tap/NXT1 pathway for nuclear export. Thus, there may be a reason(s), beyond nuclear export of intron-containing HIV-1 RNAs, that lentiviruses have evolved the use of the Rev/RRE system for nuclear export of viral RNAs through the CRM1 pathway. Presumably, the fate of viral RNAs in the cytoplasm may be subject to the pathway of nuclear export. A number of reports have indicated that HIV-1 viral particle assembly may be governed by the nuclear export pathway utilized by the viral genomic RNA (93, 94, 191, 201). All the same, replacing the Rev protein with a constitutive transport element (CTE) from Mason Pfizer monkey virus (MPMV) conferred HIV with the capacity to replicate, albeit the CTE-containing virus was dramatically attenuated (50-100 fold) compared to virus with a functional Rev protein (20). CTE-containing RNAs utilize the Tap/NXT1 nuclear export pathway, which was the same pathway employed here to examine the effect of nuclear export on Rev-enhanced RNA encapsidation. Along similar lines data presented here demonstrate that the Rev-enhanced RNA encapsidation effect does not strictly require nuclear export

through the CRM1 pathway (Figs. 18 & 19); however, the encapsidation effect was significantly attenuated compared to a functional Rev/RRE system. Although these results infer that the CRM1 pathway may partially determine the efficiency of subsequent cytoplasmic events, the use of a RevM10 mutant fused to the Tap protein may have also inadvertently decreased the efficiency of the Rev protein.

Overall it is clear that the Rev/RRE system positively influences the efficiency and specificity of late HIV-1 replication events that occur in the host cell cytoplasm. The significance of the Rev/RRE system is even more poignant when considering that these functions may be conserved among lentiviruses. Results presented here demonstrate that the RRE *cis* element and CRM1-dependent nuclear export are not necessary for Rev-mediated enhanced RNA encapsidation. However, the Rev/RRE system is apparently critical for all of these processes since in all cases efficiency was dramatically diminished when compared with the normal HIV-1 Rev/RRE system. These data insinuate that the Rev/RRE system may be a central component of a RNA encapsidation mechanism conventionally associated with *cis* elements in the 5' UTR (R, U5, PBS, and ψ). The proposition that a complete encapsidation mechanism is the concerted effect of all these components is examined in Chapter 3.

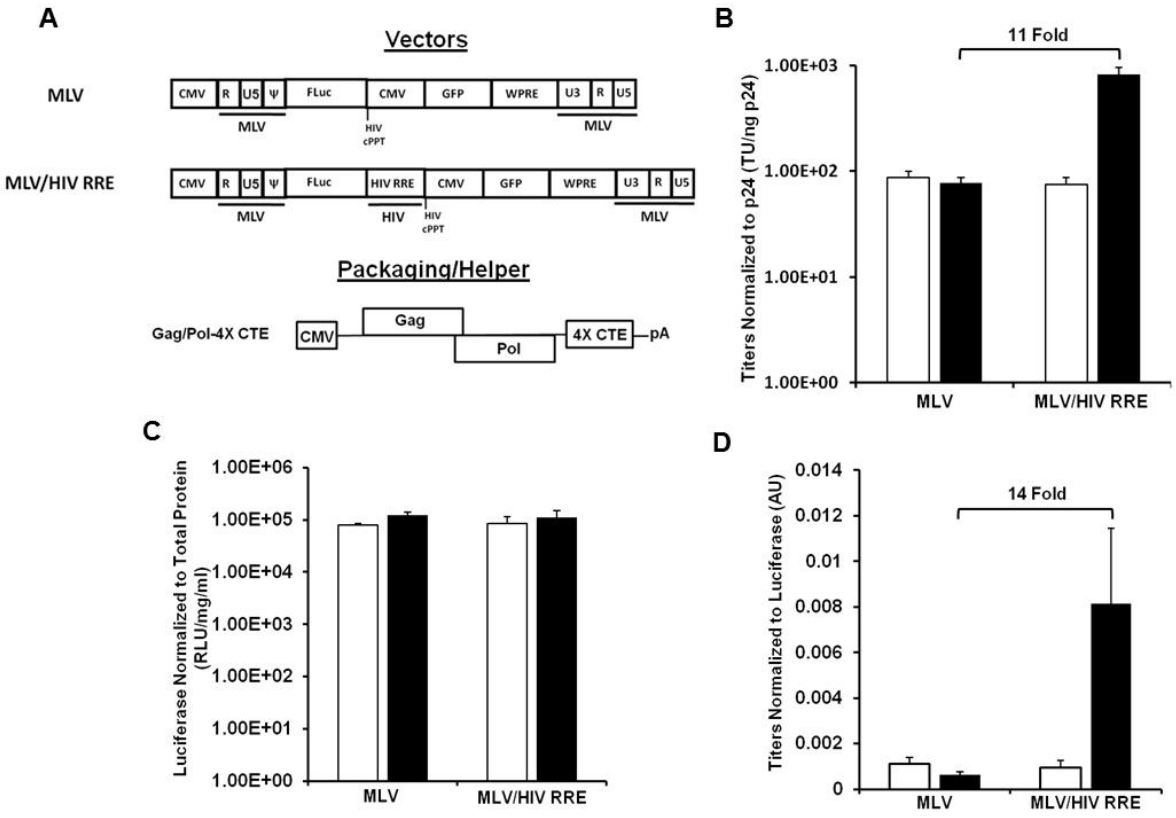


Figure 10. HIV-1 Rev/RRE system augments vector titers. A. Full-length MLV/HIV chimeric vector RNAs are expressed from a CMV (cytomegalovirus) promoter in transfected 293T cells. MLV and HIV *cis* elements can be distinguished by black underscore. Chimeric vector names are represented as MLV/HIV followed by corresponding HIV *cis* elements incorporated: RRE (Rev Response Element) and cPPT (central polypurine tract). Also incorporated are the WPRE (woodchuck hepatitis virus post-transcriptional regulatory element), FLuc (firefly luciferase gene), and GFP (green fluorescent protein gene). HIV-1 Gag-Pol 4X CTE helper construct was used to express structural and enzymatic proteins to generate viral particles independent of HIV-1 Rev protein. **B.** Vector titers normalized to p24 are shown in the absence (white bars) and presence (black bars) of Rev. The influence of adding HIV-1 *cis* elements to the MLV vector is indicated by fold increases in the presence of Rev relative to the standard MLV vector. **C.** Luciferase levels normalized to total protein are shown for each vector. **D.** Titers expressed as a ratio to luciferase are shown as arbitrary units (AU). Error for all bar graphs is expressed as ±S.D. All experiments were performed in triplicate.

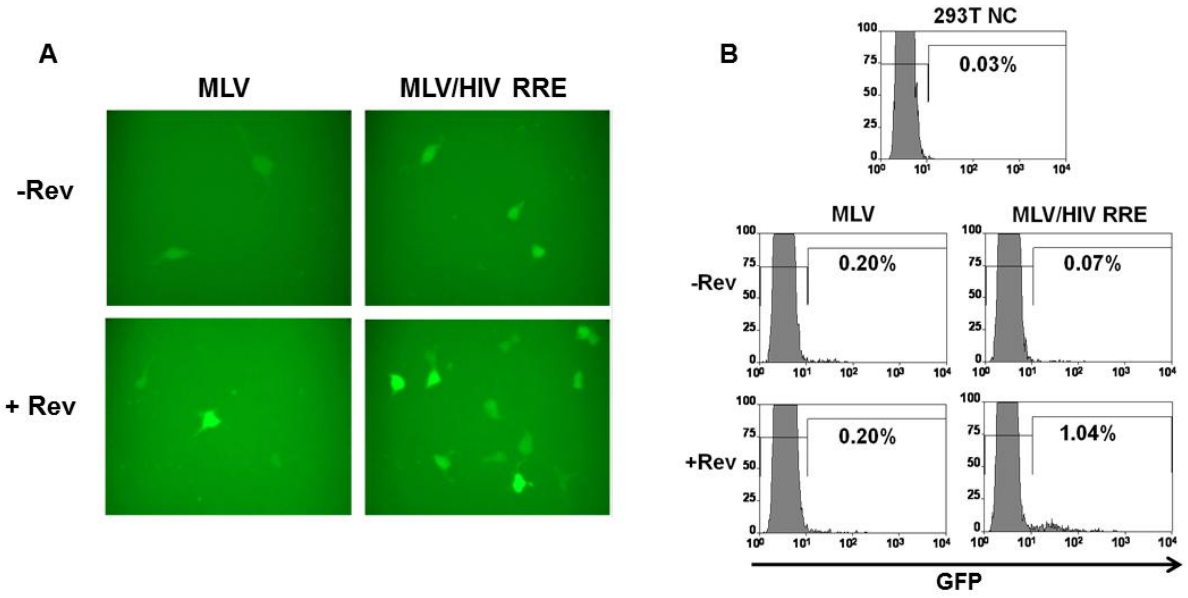


Figure 11. Transduction of 293T cells with chimeric MLV/HIV vectors packaged into HIV-1 viral particles. A and B. 293T cells were transduced with equivalent amounts of p24 capsid protein (50 ng), as determined for each of the indicated chimeric vectors. The influence of the HIV-1 Rev/RRE system on transduction was assessed by fluorescence microscopy **(A)** and FACscan analysis **(B)** at 7 days post-transduction. The percent GFP positive cells are indicated for each FACscan and 293T negative control (NC) cells are shown.

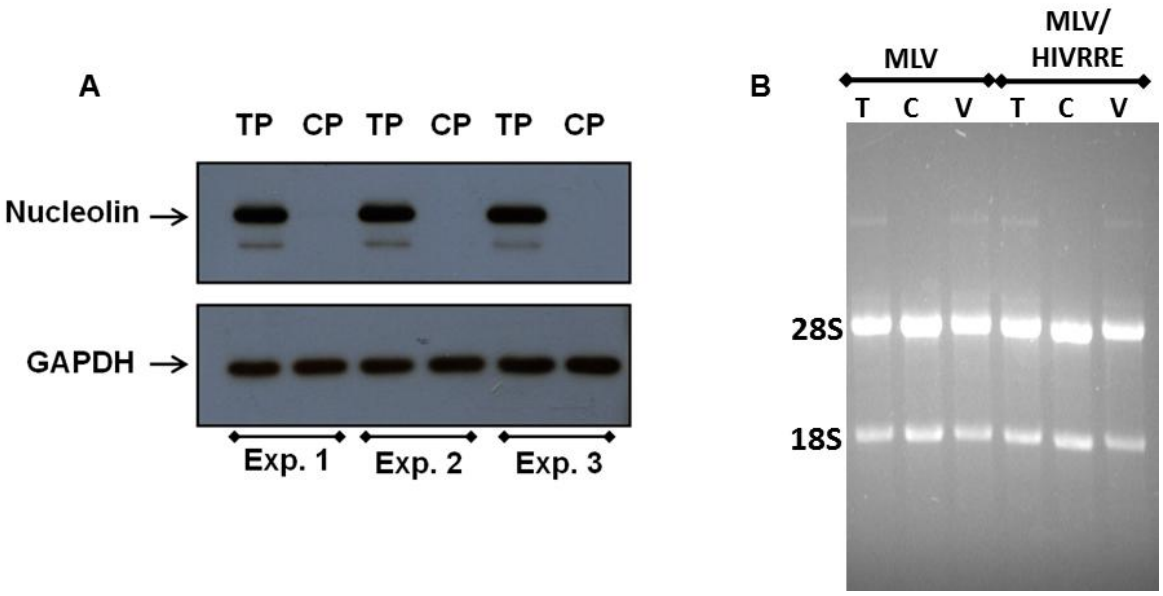


Figure 12. Cytoplasmic separation and RNA integrity. A. At the time of harvesting vectors total and cytoplasmic protein fractions were routinely collected from the producer 293T cells to monitor separation of the cytoplasmic fraction. Equivalent amounts of total (TP) and cytoplasmic (CP) protein lysates were analyzed for nucleolin (a nuclear specific protein), and GAPDH, by western blot analysis. Three independent experiments are shown for the MLV/HIV RRE+RU5PS vector (described in chapter 3) packaged into HIV-1 particles in the presence of Rev. **B.** Total (T), cytoplasmic (C), and viral particle (V) RNAs were routinely monitored for integrity by resolving equivalent amounts of RNA on a denaturing (foramaldehyde) 1.2% agarose gel, stained with ethidium bromide. The 28S and 18S ribosomal bands are shown. At the time of RNA isolation viral particle RNA is copurified with cellular RNA, hense the dominant ribosomal bands in the viral particle RNA lanes.

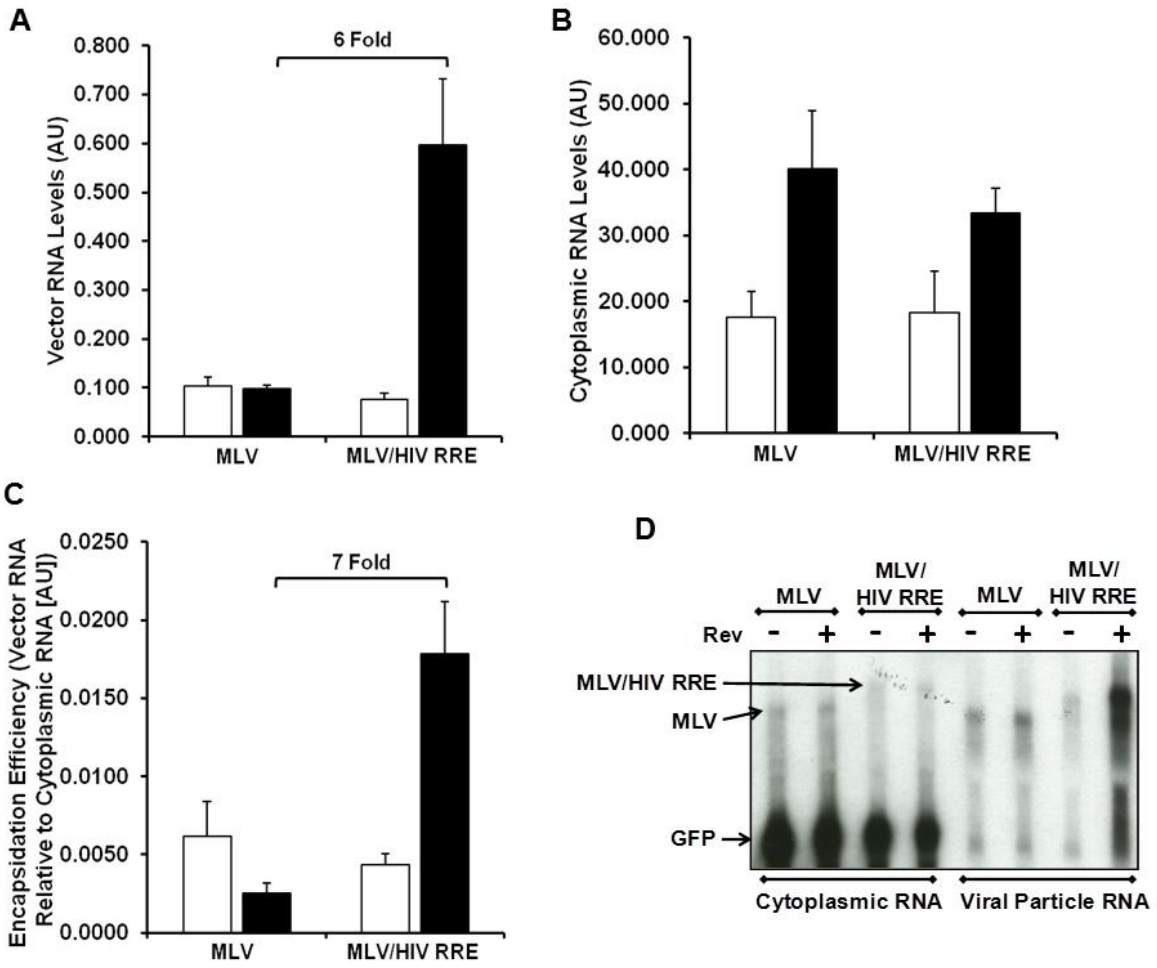


Figure 13. HIV-1 Rev/RRE enhances encapsidation of chimeric MLV/HIV RNA into HIV-1 viral particles. A. Vector RNA was measured by qRT-PCR and expressed in arbitrary units (AU). RNA levels for all graphs are shown in the absence (white bars) and presence (black bars) of Rev. The influence of adding HIV-1 *cis* elements to the MLV vector is indicated by fold increases in the presence of Rev relative to the standard MLV vector. **B.** Cytoplasmic RNA was isolated from vector producer 293T cells at the time of vector harvesting. Relative RNA levels were obtained and recorded as done for vector RNA in part A. **C.** Efficiency of encapsidating RNA into HIV-1 viral particles is expressed as a ratio of vector RNA in viral particles to cytoplasmic RNA available for encapsidation. Relative levels are expressed like vector RNA in part A. **D.** Northern blot analysis of cytoplasmic and vector RNA isolated from MLV and MLV/HIV RRE in the absence (-) and presence (+) of Rev. Vector length RNA species were detected with a GFP labeled probe, as well as an additional RNA species (labeled GFP) generated from the internal CMV promoter.

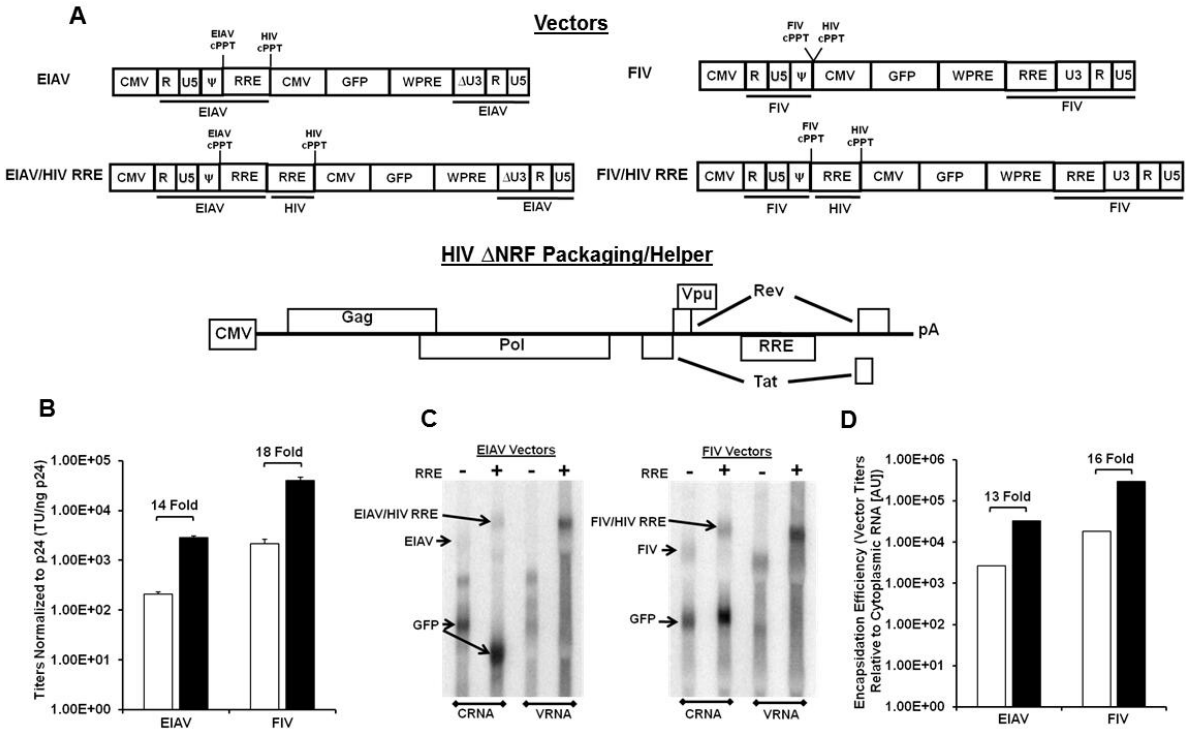


Figure 14. HIV-1 Rev/RRE enhances encapsidation of chimeric EIAV/HIV and FIV/HIV RNAs into HIV-1 viral particles. A. Full-length EIAV/HIV and FIV/HIV chimeric vector RNAs are expressed from a CMV (cytomegalovirus) promoter in transfected 293T cells. EIAV or FIV, and HIV *cis* elements can be distinguished by black underscore. Chimeric vector names are represented as EIAV/HIV, or FIV/HIV, followed by corresponding HIV *cis* elements incorporated: RRE (Rev Response Element) and cPPT (central polypurine tract). Also incorporated are the WPRE (woodchuck hepatitis virus post-transcriptional regulatory element), and GFP (green fluorescent protein gene). HIV-1 ΔNRF helper construct was used to express structural and enzymatic proteins to generate viral particles, which ectopically supplies Rev. **B.** Vector titers normalized to p24 are shown in the absence (white bars) and presence (black bars) of the RRE. The influence of adding HIV-1 *cis* elements to the EIAV, or FIV, vector is indicated by fold increases in the presence of Rev relative to the standard EIAV, or FIV, vector. **C.** Northern blot analysis of cytoplasmic and vector RNA isolated from EIAV, or FIV, and EIAV/HIV RRE, FIV/HIV RRE, in the absence (-) and presence (+) of RRE. Vector length RNA species were detected with a GFP labeled probe, as well as an additional RNA species (labeled GFP) generated from the internal CMV promoter. **D.** Efficiency of encapsidating RNA into HIV-1 viral particles was indirectly expressed as a ratio of vector titer to cytoplasmic RNA available for encapsidation, and expressed as arbitrary units (AU). Error (where indicated) is expressed as ±S.D.

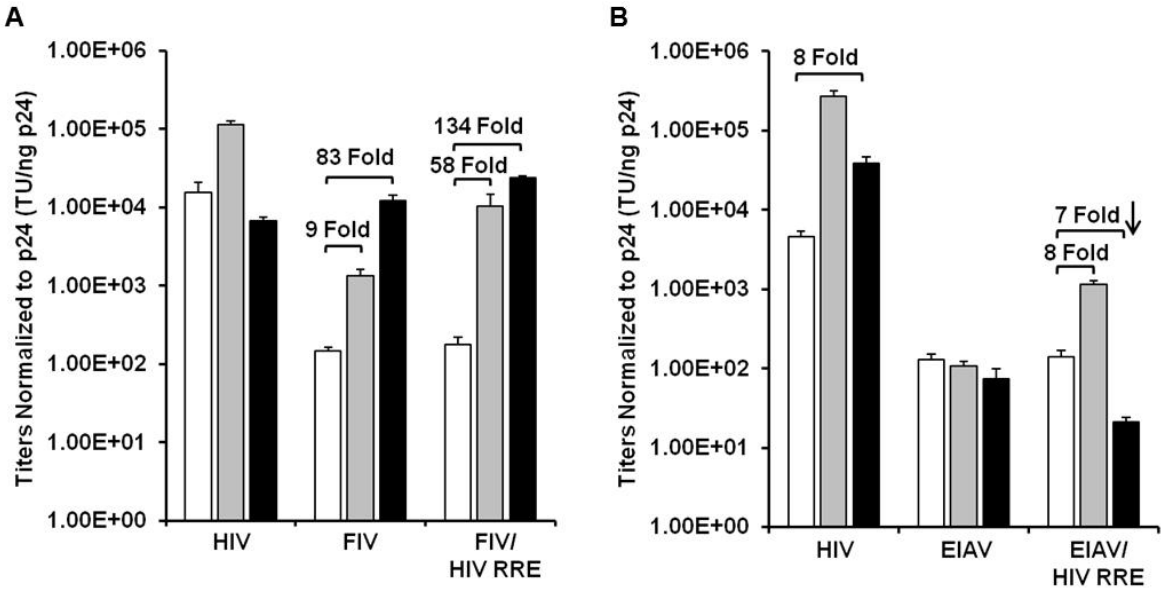


Figure 15. FIV Rev, but not EIAV Rev, augment titers of chimeric vectors packaged into HIV-1 viral particles. Vector titers normalized to p24 are shown in the absence (white bars) of any Rev, in the presence of HIV Rev (gray bars), and in the presence of FIV Rev (black bars, A) or EIAV Rev (black bars, B). **A.** Normalized titers were assessed for HIV, FIV, and FIV/HIV RRE chimeric vectors packaged into HIV-1 derived viral particles. **B.** Normalized titers were measured for HIV, EIAV, and EIAV/HIV RRE chimeric vectors packaged into HIV-1 derived viral particles.

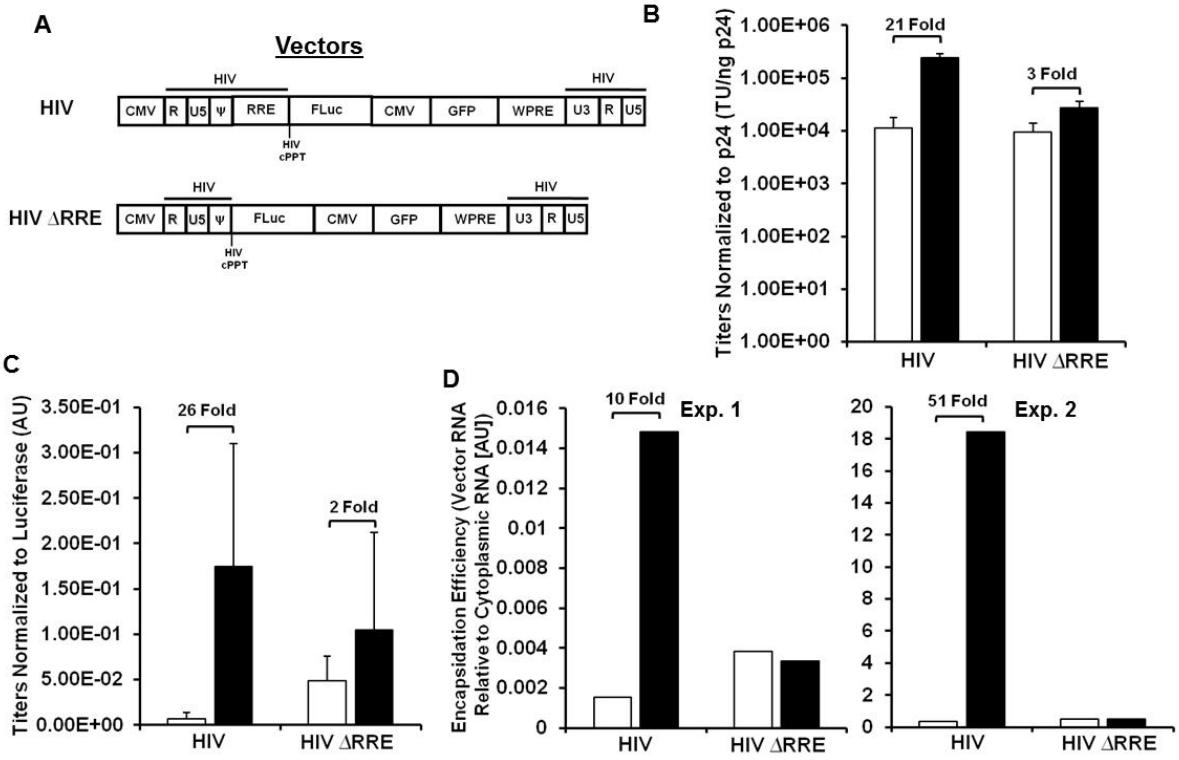


Figure 16. HIV-1 Rev is dependent upon the RRE to augmet RNA encapsidation into HIV-1 viral particles. A. Full-length HIV-1 vector RNAs are expressed from a CMV (cytomegalovirus) promoter in transfected 293T cells. HIV *cis* elements are indicated by black underscore. Also incorporated are the WPRE (woodchuck hepatitis virus post-transcriptional regulatory element), FLuc (firefly luciferase gene), and GFP (green fluorescent protein gene). The RRE was removed from the HIV ΔRRE construct. HIV-1 Gag-Pol 4X CTE helper construct was used to express structural and enzymatic proteins for generation of viral particles independent of HIV-1 Rev protein. **B.** Vector titers normalized to p24 are shown in the absence (white bars) and presence (black bars) of Rev. The influence of removing the RRE from the HIV-1 vector is indicated by the fold increase in the presence of Rev. **C.** Titers expressed as a ratio to luciferase are shown as arbitrary units (AU). **D**. Efficiency of encapsidating RNA into HIV-1 viral particles is expressed as a ratio of vector RNA in viral particles to cytoplasmic RNA available for encapsidation. Two independent experiments are shown. Relative levels are expressed as arbitrary units (AU). All experiments were executed in the absence (white bars) and presence (black bars) of Rev. Error for bar graphs is expressed as ±S.D.

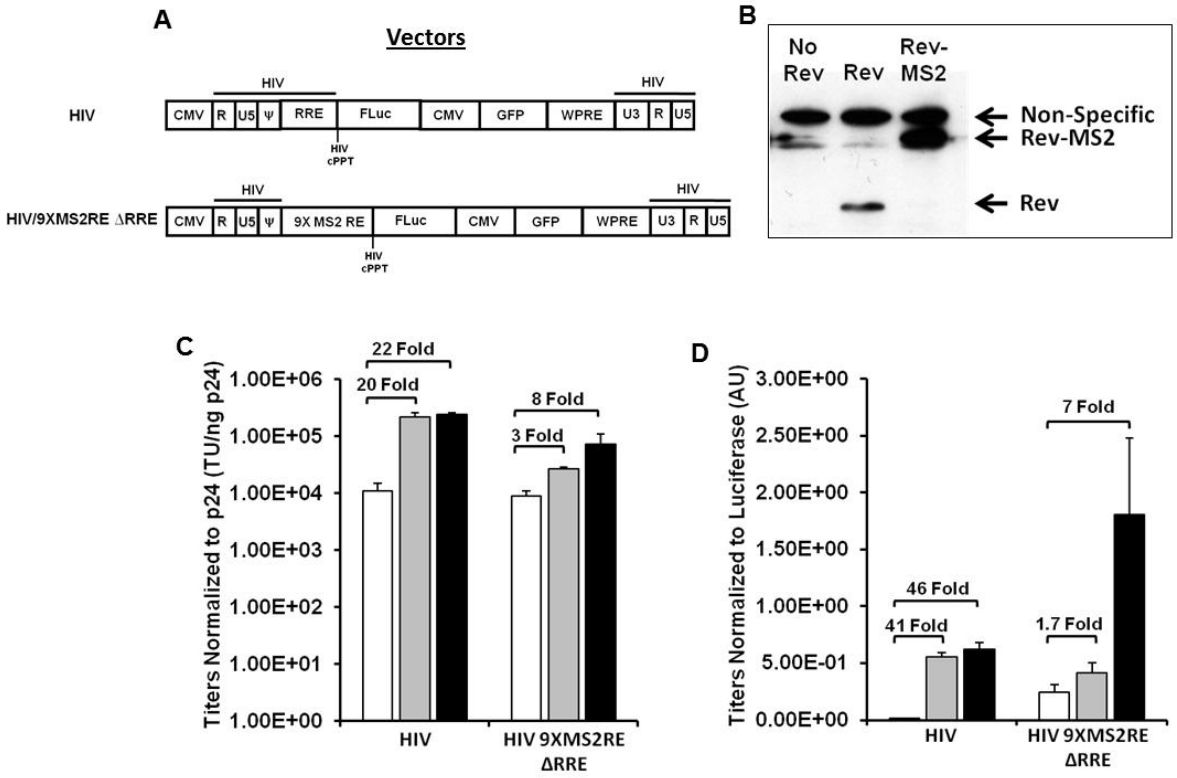


Figure 17. Rev-enhanced encapsidation in the absence of the HIV-1 RRE. A. The HIV-1 RRE *cis* element was replaced with nine tandem copies of the MS2 response element derived from the MS2 bacteriophage to generate the HIV/9XMS2RE ΔRRE vector. **B.** Western blot analysis of Rev (E116D) and Rev-MS2 fusion protein. Proteins were identified with a mouse anti-Rev antibody. **C.** Vector titers normalized to p24 are shown in the absence of Rev(white bars), in the presence (gray bars) of Rev (E116D), or in the presence of the Rev-MS2 fusion protein. The influence of Rev and Rev-MS2 are indicated by fold increases relative to the no Rev. **D.** Titers expressed as a ratio to luciferase are shown as arbitrary units (AU). Error for all bar graphs is expressed as ±S.D.

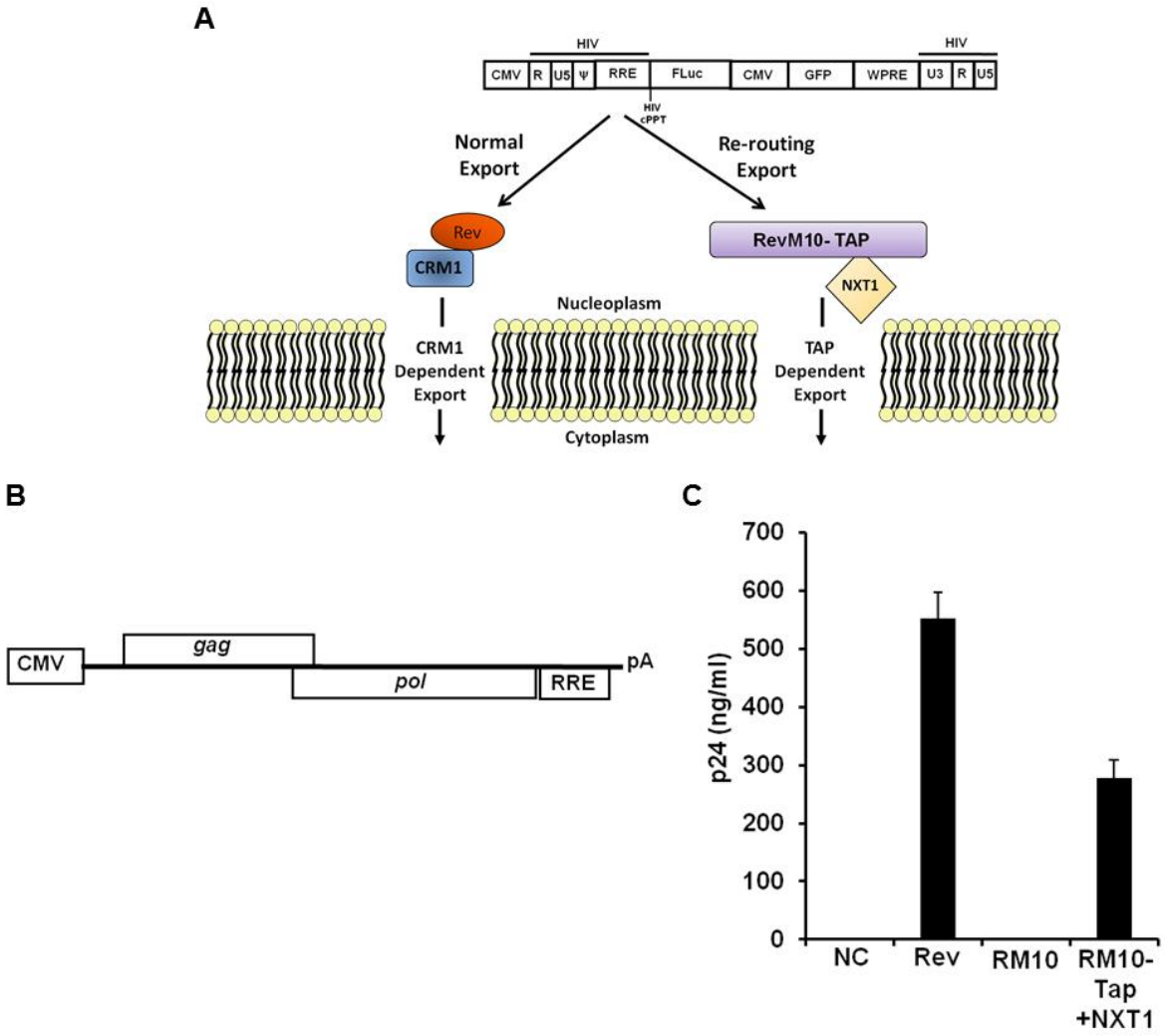


Figure 18. Rev-dependent RNAs can be redirected from CRM1 nuclear export to the TAP/NXT1 nuclear export pathway. A. HIV-1 derived viral RNA is redirected from nuclear export through the CRM1-dependent pathway to the TAP/NXT1 pathway. **B.** To test the system an RRE-dependent *gag/pol* packaging construct was used, where p24 expression is only achieved in the presence of Rev. **C.** The concentration of p24 was measured in the media of 293T cells cotransfected with the packaging construct in the absence of Rev (negative control, NC), or in the presence of HIV-1 Rev; RevM10 (RM10, a Rev mutant that lacks the capacity for nuclear export); or, coexpression of RevM10-Tap fusion in the presence of NXT1 (RevM10 mutant maintains RNA interaction and precludes nuclear export via CRM1, while redirecting RNA through Tap/NXT1 pathway).

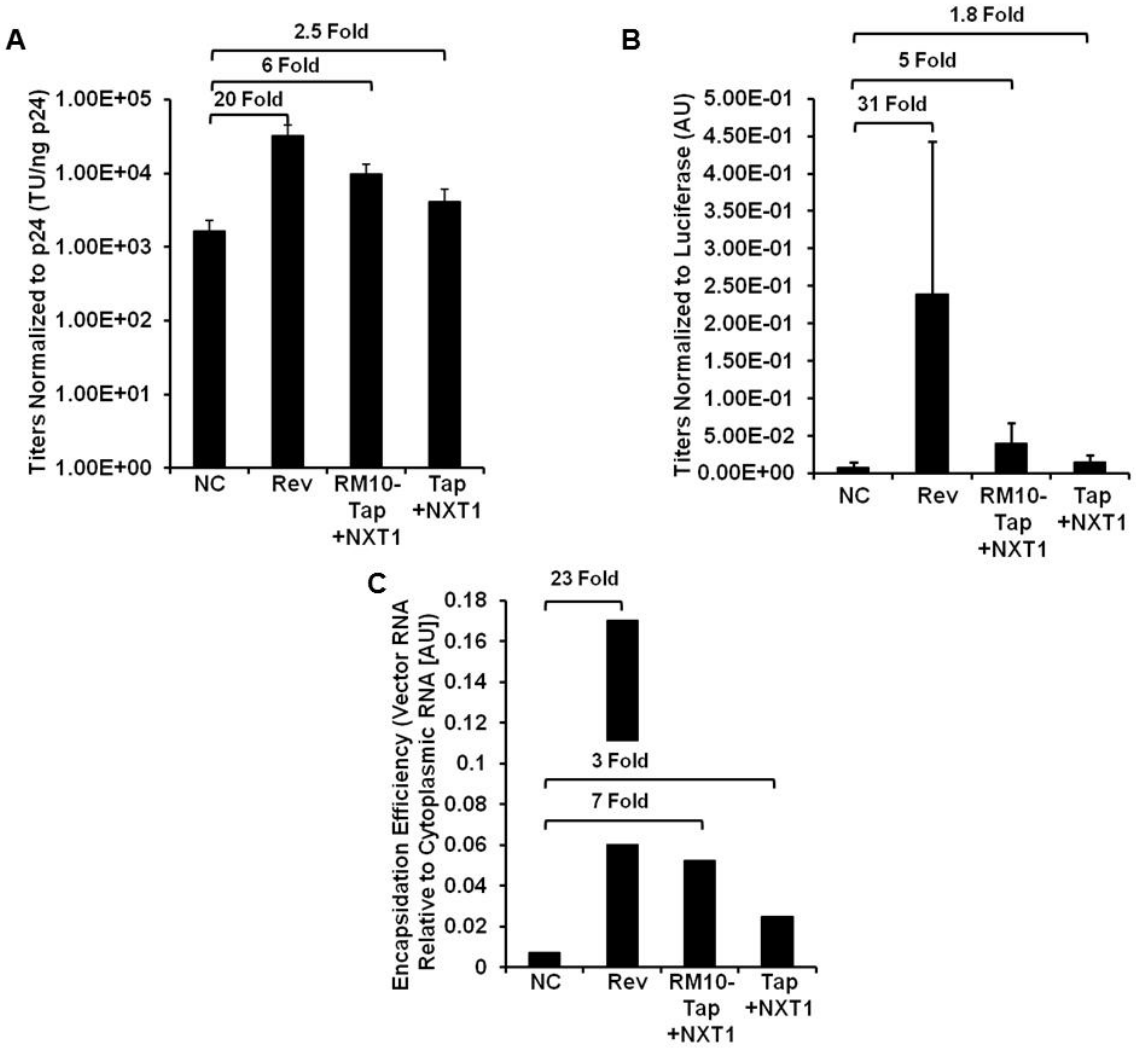


Figure 19. HIV-1 Rev can enhance RNA encapsidation through the Tap/NXT1 nuclear export pathway. A. Vector titers normalized to p24 are shown in the absence of Rev (NC) or in the presence of Rev; RevM10-Tap + NXT1; and Tap + NXT1. **B.** Titers expressed as a ratio to luciferase are shown as arbitrary units (AU). **C.** Efficiency of encapsidating RNA into HIV-1 viral particles is expressed as a ratio of vector RNA in viral particles to cytoplasmic RNA available for encapsidation. For all graphs the influence of each nuclear export factor is expressed as fold increase relative to the negative control. Error for bar graphs is expressed as ±S.D.

Chapter 3

HIV-1 CIS ELEMENTS IN THE 5' UTR AND REV/RRE SYSTEM ARE REQUIRED FOR EFFICIENT AND SPECIFIC PACKAGING OF RNA INTO HIV-1 PARTICLES[1]

Introduction

The Rev/RRE system positively impacts encapsidation of heterologous RNA into HIV-1 viral particles in the absence of conventional HIV-1 *cis* elements (Chapter 2). However, it is well established that encapsidation is primarily mediated through *cis* elements in the 5' UTR, predominantly protein-RNA interactions of the nucleocapsid with the conventional packaging signal, ψ (49, 149), and RNA-RNA dimerization via stem-loop 1 of ψ (49, 148). The conventional canonical *cis* packaging signal is a ~120 nucleotide fragment comprised of four stem-loop structures located in the HIV-1 5' untranslated region (UTR), and extending into the 5' end of the HIV-1 Gag coding sequence (4, 36, 49, 120, 138, 139). Interactions of the Gag polyprotein with stem-loops 2, 3, and 4 ensure efficient encapsidation of the gRNA (49, 149). In addition to ψ, several reports indicate that *cis* elements in the HIV-1 5' R, U5, and PBS regions can also influence HIV-1 RNA packaging (37, 38, 81, 139). Apparently, the HIV packaging system is highly complex comprising multiple components that may function at different steps of a comprehensive packaging mechanism.

In general, the components that support efficient and specific lentivirus RNA packaging are not defined by a contiguous *cis* RNA element that can confer packaging onto a

[1]A portion of the work in this chapter was published by Adam Cockrell, Henriette van Praag, Nicholas Santistevan, Hong Ma, and Tal Kafri, titled:The HIV-1 Rev/RRE system is required for HIV-1 5' UTR *cis* elements to augment encapsidation of heterologous RNA into HIV-1 viral particles (45).

heterologous RNA (15). Lentivirus *cis* packaging signals have predominantly been defined by mutagenesis, genetic, and functional studies. The most highly studied lentivirus packaging signal is that from HIV-1, with a number of different *cis* elements defined in the 5' UTR as described above. Multiple *cis* elements, functioning as a composite packaging signal in the 5' UTR, appears to be a feature conserved among lentiviruses, as indicated by data for HIV-2 (63, 140), SIV (170, 197), and FIV (21, 103, 183). A number of lentivirus studies indicate that disparate *cis* elements from the 5' UTR of a single viral genomic RNA may function through intramolecular interactions (50, 118, 183, 214). An innovative biochemical technology called SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension) produced high resolution secondary RNA structures for the 5' UTRs of HIV-1 *in virio* and *ex virio* (212, 214) (Fig. 8 from chapter 1) and FIV from *in vitro* transcribed RNA (104). These structures revealed long-range intramolecular RNA-RNA interactions between *cis* elements, which may be indicative of function. Conceivably, the concerted effort of multiple *cis* elements from the R, U5, PBS, and ψ regions of the 5' UTR may be important for specific and efficient encapsidation.

The *cis* packaging signals for simple retroviruses, on the other hand, appear to be distinct from lentiviruses in that defined, contiguous *cis* elements from the 5' UTR are sufficient to mediate encapsidation. The murine leukemia virus (MLV) harbors a *cis* element of ~175 nucleotides, primarily in the 5' UTR that can readily support retroviral RNA packaging if relocated to a different position within homologous RNA (134), and can bestow efficient packaging upon heterologous RNA (3, 83). Similarly, a ~160 nucleotide packaging signal for Rous sarcoma virus was sufficient to confer nearly 100% packaging efficiency for a heterologous RNA (12, 13). Furthermore, the R and U5 regions of the 5' UTR from simple

retroviruses are not part of the transferrable packaging signal. Therefore, packaging systems for simple retroviruses are primarily comprised of a small contiguous *cis* element in the viral genomic RNA that can select the RNA from the milieu of host cell RNAs for encapsidation.

Similar to simple retroviral packaging systems, it is anticipated that a genuine HIV-1 packaging system could be expected to have *cis* elements and *trans* factors that impart a gain-of-function onto a foreign RNA for specific and efficient encapsidation into HIV-1 viral particles. As alluded to above, different studies indicate that efficient and specific RNA encapsidation into HIV-1 viral particles rely upon multiple components comprised of both *cis* elements in the RNA (R, U5, PBS, ψ, and RRE) and viral encoded *trans* factors (Gag protein and Rev). However, it is not understood if the RNA encapsidation mechanism is a single pathway relying upon the concerted effects of the various components. Studies from chapter 2 were expanded whereby the 5' UTR *cis* elements and Rev/RRE system were systematically reconstructed in the middle of a simple retroviral vector RNA (derived from MLV) to investigate the collective impact on the gain of encapsidation function into HIV-1 derived viral particles. The data demonstrate that: i) the HIV-1 Rev/RRE system is required for *cis* elements from the 5' UTR to mediate encapsidation of a heterologous RNA; ii) the complete contingent of *cis* elements from the 5' UTR (R, U5, PBS, and ψ) are required for encapsidation of a heterologous RNA; iii) a functional HIV-1 packaging system is composed of multiple components (including 5'UTR *cis* elements, nucleocapsid, and the Rev/RRE system); iv) the HIV-1 packaging system specifically selects for RNA encapsidation into HIV-1 viral particles, but does not influence encapsidation into MLV viral particles; and v) specific encapsidation of RNA mediated by the HIV-1 packaging system reduces nonspecific packaging of cellular RNAs. Additionally, the influence of *cis* elements from the canonical

packaging signal was further substantiated in the presence of the Rev/RRE system through mutational analysis of HIV-1 vector RNAs.

Methods

Plasmid Constructs. Murine leukemia virus (MLV) and MLV/HIV RRE chimeric vector constructs were generated as described in chapter 2 methods. The MLV/HIV RU5PS chimeric vector (pTK1442) was generated by subcloning a *BamHI* fragment into the *BamHI* site of pTK1328. The *BamHI* fragment was derived from a PCR amplified product originally cloned into pCR2.1-TOPO (Invitrogen) (pTK1428) and moved into the *SmaI/Acc65I* sites of pBlueScript (Strategene) (pTK1441). The PCR amplified product, comprising the HIV-1 R, U5, PBS, and canonical packaging signal which extends into the 5' end of Gag, was generated with the following primers: HIV RU5 For 5'-gcggccgcttaattaagggtctctctggttagaccagatctgagcc-3'; and HIV RU5Pack. Rev 5'-gcggccgcttgctgtgcgg-3'. The MLV/HIV RRE+RU5 vector (pTK1439) was constructed by subcloning a PCR amplified fragment into the *NotI* site of pTK1332. The PCR amplified product was formally cloned into pCR2.1-TOPO (Invitrogen) (pTK1427). The following primers were used to generate a PCR product comprising the HIV-1 R and U5 regions: HIV RU5 For primer as described above and HIV RU5 Rev 5'-gcggccgcactgctagagattttccacactgac-3'. The MLV/HIV RRE+PS vector (pTK1423) was constructed by subcloning a fragment, consisting of the canonical packaging signal and extending into the 5' end of Gag and HIV-1 RRE, into the *BamHI* sites of pTK1332. Upon cutting with *BamHI* the existing RRE in pTK1332 would not be retained. The last construct in the series, the MLV/HIV RRE+RU5PS vector (pTK1440), was generated by subcloning

the *NotI* fragment from pTK1428 into the *NotI* site of pTK1332. The *NotI* fragment contains the HIV-1 R, U5, PBS, and canonical packaging signal extending into the 5' end of Gag.

The standard HIV-1 derived vectors are described in chapter 2 methods. The following is a description of how HIV-1 vectors with deletions in the canonical packaging signal (ψ) were generated. The following cloning series was used to generate the HIV-1 Δ ψ vector: i) a *MluI/NotI* fragment, containing the 5'UTR from the HIV-1 vector pTK1087 was subcloned into pSL301 (Invitrogen) to generate pTK1221; ii) pTK1221 was used as a template for PCR around the entire plasmid wherein primers were designed to eliminate the canonical packaging signal, the region between the *MluI/NotI* sites was confirmed by sequencing to be correct with elimination of the canonical packaging signal, pTK1230; iii) the *MluI/NotI* fragment was subcloned into pTK1087 to generate pTK1240; and iv) the firefly luciferase gene was inserted into the *EcoRI* site of pTK1240 to produce the final construct, pTK1305 containing a deletion of the entire canonical packaging signal. The HIV-1 Δ SL1 construct was generated by the following cloning series: i) pTK1221 was used as a template for PCR around the entire plasmid wherein primers were designed to eliminate stem-loop 1, the region between the *MluI/NotI* sites was confirmed by sequencing to be correct with elimination of stem-loop 1, pTK1426; and, ii) the *MluI/NotI* fragment was subcloned into pTK1363 to generate the final construct, pTK1436 with a deletion of stem-loop 1. The HIV-1 Δ SL1,3,4 produced with the following cloning series: i) pTK1221 was used as a template for PCR around the entire plasmid wherein primers were designed to eliminate stem-loops 3 & 4, the region between the *MluI/NotI* sites was confirmed by sequencing to be correct with elimination of stem-loops 3 & 4, pTK1424; ii) pTK1424 was used as a template for PCR around the entire plasmid wherein primers were designed to

eliminate stem-loop 1, the region between the *MluI/NotI* sites was confirmed by sequencing to be correct with elimination of stem-loops 1, 3, & 4, pTK1429; iii) the *MluI/NotI* fragment was subcloned into pTK1363 to generate the final construct, pTK1437 with a deletion of stem-loops 1, 3, & 4.

The packaging constructs supplying necessary structural/enzymatic proteins were 4XCTE or ΔNRF as described in chapter 2 methods. The Rev and VSV-G expression constructs are explained in chapter 2 methods.

Cells, Viral Particle Production, and Concentration. All are described in chapter 2 methods. Regarding experiments executed for packaging HIV-1 vector RNAs that were deleted of the entire canonical packaging signal (pTK1305); vector production for these experiments required cotransfection of 5μg Tat expressing plasmid. In addition, the SODk0 cells were used for experiments in which the absence of large T antigen was pertinent. These cells are derived from 293 cells, but lack the large T antigen, and are maintained in the same media as 293T cells, as described in chapter 2 methods.

HIV-1 p24 Capsid Concentration. Details of this assay were described in chapter 2 methods.

MLV Reverse Transcriptase Assay. MLV reverse transcriptase activity was executed on vector particles (5 μl) harvested from the media by mixing with 25 μl of RT activity reagent (60mM Tris pH8, 0.6mM $MnCl_2$, 90mM KCl, 0.125mg/ml Poly A [Roche], 6 μg/ml oligo dT_{16}, 25mM DTT, 0.06% Triton X-100, and 0.25 mCi/ml ^{3}H-TTP [MP Biomedicals]). After incubation for 1 hour at 37°C the entire volume was spotted onto DE81 anion exchange chromatography paper (Whatman), and placed into 5% Na_2PO_4 for 5 minutes at room temperature. The samples were washed at room temperature five times, for 5 minutes each,

with 5% Na_2PO_4 two times with water, and one time with 95% ethanol. The samples were dried, placed in scintillation fluid, and radioactivity was measured on a Beckman LS 6500 scintillation counter. Values were recorded as CPM. Data used for titer normalization, and determining amount of viral particles for RNA isolation, are a mean of replicate samples.

FACS Analysis. Details of this assay were described in chapter 2 methods.

Luciferase Assays. Details of this assay were described in chapter 2 methods.

RNA Isolation. RNA was isolated as described in chapter 2 methods.

qRT-PCR. Quantitative RT-PCR was executed as described in chapter 2 methods. Additional primer-probe sets used to obtain data were directed to the large T antigen: LT-63 For 5'-tgcaaggagtttcatcctga-3', LT-63 Rev 5'-ggttgatgagcatattttactcca-3', and probe #63 (Roche Universal Probe Library). Primer-probe set used for HIV-1 vector data in figure 31 were directed to the RRE cis element: RRE For 5'-agcatctgttgcaactcacagt-3', RRE Rev 5'-tttccacagccaggattctt-3', and probe #148 (Roche Universal Probe Library).

Northern Blot Analysis. Northern blots were executed as described in chapter 2 methods. However, the probe used in figure 24 was a BstEII fragment from pTK1440 generated to the 5' end of the vector RNA.

Real Time qPCR. 293T cells were transduced with 1000ng p24 from concentrated HIV-1 vector packaged into HIV-1 viral particles. Total cellular DNA was isolated at 24 hours after transduction of 293T cells. To collect total DNA transduced cells were lysed in a proteinase K solution (10 mM Tris pH 8, 10 mM EDTA pH 8, 0.5% SDS, 0.4 M NaCl, and 200 µg/ml proteinase K) for 48 hours at 55°C. Total DNA was extracted with v/v phenol (Invitrogen) and v/v phenol/chloroform/isoamyl alcohol (Invitrogen) and then treated with RNase A (20

μg; Fermentas) at 37°C for 2 hours. A second round of extractions was performed and total DNA was precipitated using standard ethanol precipitation.

The relative levels of large T antigen cDNA were normalized for β-globin levels. qPCR for β-globin was described previously (100). The primer-probe set for large T antigen is as described above in qRT-PCR section. The relative levels were quantitated using the $2^{-\Delta Ct}$ method as described in chapter 2 methods.

Results

The HIV-1 Rev/RRE system is required for *cis* elements from the 5' UTR to mediate efficient RNA encapsidation into HIV-1 viral particles. Results in chapter 2 demonstrate that the Rev/RRE system may be part of the encapsidation mechanism. However, it is well established that encapsidation is primarily mediated through *cis* elements in the 5' UTR, predominantly protein-RNA interactions of the nucleocapsid with (49, 149), intermolecular RNA-RNA dimerization via stem-loop 1 of (49, 148), and presumably intramolecular RNA-RNA interactions (50, 118, 183, 214). The HIV-1 Rev/RRE, as well as the aforementioned protein-RNA and RNA-RNA interactions may comprise a *bona fide* packaging system that may be defined by the capacity to support efficient and specific encapsidation of a heterologous RNA into HIV-1 derived viral particles. To characterize the role of the Rev/RRE in the context of a packaging system that comprises the 5' UTR *cis* elements we generated a series of heterologous MLV/HIV vectors (Fig.20A). The entire 5' UTR was either independently incorporated into the heterologous MLV RNA (MLV/HIV RU5PS), or inserted in the context of the RRE (MLV/HIV RRE+RU5PS). A diagram of the 5' UTR is depicted in Figure 20B. Similar to vectors in the results section of chapter 2, heterologous

vector RNAs were packaged into viral particles generated from a helper system (Gag/Pol-4X CTE) that does not rely upon the Rev/RRE system for nuclear export of RNA, encoding the structural and enzymatic proteins (Fig. 20A). Since *cis* elements in the R and U5 regions have also been shown to impact encapsidation (37, 38, 81, 139), the 5' UTR was also further separated into the R/U5 (MLV/HIV RRE+RU5) and ψ (MLV/HIV RRE+PS) in the context of the RRE (Fig. 20A). Each of the vectors was titered on 293T cells by scoring for GFP positive cells, and normalized to levels of p24 capsid protein.

The complete contigent of *cis* elements from the 5' UTR (MLV/HIV RU5PS) moderately enhanced titers (12 fold) independent of Rev, whereas in the context of the RRE (MLV/HIV RRE+RU5PS) Rev dramatically augmented titers (626 fold) compared to the basic MLV vector (Fig. 21A); an effect that was visually, and quantitatively, outstanding upon transduction of 293T cells with equivalent amounts of p24 capsid protein (Fig. 22A & B). Notably, titers of the MLV/HIV RRE+RU5PS vector were $1.07x10^7$ TU/ml, which is in the 10^7-10^8 TU/ml range of those obtained with standard HIV-1 vectors prior to concentration; a comparison that was also observed after normalization to p24. These data indicate that the Rev/RRE system and 5' UTR *cis* elements synergize to achieve the increase in vector titer. Furthermore, to confirm that GFP titers are a result of reverse transcription of heterologous MLV vector RNA by the HIV-1 reverse transcriptase, transduction of 293T cells was assessed by FACs analysis and fluorescence microscopy following treatment with the HIV-1 specific non-nucleoside RT inhibitor, etravirine (Fig. 23A). Etravirine specifically inhibited transduction of the MLV/HIV RRE+RU5PS vector packaged into HIV-1 viral particles, but did not inhibit transduction of the same vector packaged into MLV viral particles (Fig. 23A). As a positive control, a similar effect was observed upon transduction of

293T cells with a HIV-1 vector (Fig. 23B). Importantly, these data clearly indicate that GFP expression is not a consequence of pseudotransduction, but is dependent upon HIV-1 directed reverse transcription of the RNA packaged into viral particles. Although Rev enhanced titers of the above mentioned vectors containing the RRE, cytoplasmic luciferase levels remained relatively similar, indirectly indicating that increased titers were probably not a consequence of increased nuclear export of vector RNA (Fig. 21B & C). The titers were a clear indication that a comprehensive HIV-1 packaging system may comprise the synergistic influences of the Rev/RRE system and *cis* elements from the 5' UTR, as well as nucleocapsid protein. Considering this possibility, we sought to directly characterize the Rev impact on the encapsidation efficiency of heterologous RNAs, containing *cis* elements from the 5' UTR, into HIV-1 viral particles. Quantitative RT-PCR was employed to quantify vector RNA in viral particles, and in producer cells.

Surprisingly, as shown in Fig. 24A and C, in contrast to the increase in vector titers (Fig. 21A), incorporation of the 5' UTR *cis* elements into the heterologous MLV vector (MLV/HIV RU5PS) did not enhance RNA encapsidation in either the absence or presence of Rev. Most importantly, in the context of the RRE (MLV/HIV RRE+RU5PS), however, the 5' UTR *cis* elements exhibited a 22 fold increase in heterologous vector RNA encapsidation into HIV-1 viral particles in the presence of Rev (Fig. 24C). Rev-dependent encapsidation was clearly a consequence of enhanced RNA packaged into viral particles (Fig. 24A), not increases in cytoplasmic RNA (Fig. 24B). These data demonstrate that the Rev-RRE interaction may initially be required to render the RNA amenable for subsequent steps in the encapsidation mechanism that conventionally involve *cis* elements from the 5' UTR, such as interaction between nucleocapsid and the canonical packaging signal. The Rev-dependent

enhancement of MLV/HIV RRE+RU5PS vector RNA encapsidation obtained by qRT-PCR was bolstered by northern blot analysis showing strong Rev-dependent increases in levels of RNA encapsidated into HIV-1 viral particles, despite nominal changes in cytoplasmic vector RNA levels (Fig. 24D). Notably, within the cytoplasmic RNAs our probe detects a dominant smaller species of vector RNA (termed 'partial vector RNA'). Although the full-length vector RNA is present at lower levels in the cytoplasm, the Rev/RRE system and 5' UTR *cis* elements impart the ability of the full-length vector RNA to out compete the more abundant 'partial vector RNA' species for packaging into viral particles (Fig. 24D); demonstrating the specificity that these components confer upon a RNA for encapsidation.

Further separation of the 5' UTR *cis* elements into the canonical packaging signal (MLV/HIV RRE+PS) or R/U5 (MLV/HIV RRE+RU5) did not approach titer/luciferase (Fig. 25A), or RNA encapsidation (Fig. 25B) levels observed with the vector containing the entire 5' UTR and RRE (22 fold, Fig. 24), demonstrating the significance of retaining a fully intact 5' UTR. Moreover, the enhanced encapsidation effect of the MLV/HIV RRE+RU5PS chimeric vector is dependent upon the complete contingent of 5' UTR *cis* elements, since dissection of the 5' UTR *cis* elements into the canonical packaging signal (MLV/HIV RRE+PS) or RU5 (MLV/HIV RRE+RU5) did not result in Rev-dependent increases in encapsidation efficiency beyond the 6 fold increase obtained with the MLV/HIV RRE vector (Fig. 25B).

Overall, these data demonstrate that: i) Rev is required for efficient encapsidation of a heterologous RNA that is subsequently mediated by RNA-RNA and RNA-protein interactions through *cis* elements in the 5' UTR; ii) the complete contingent of *cis* elements from the 5' UTR (R, U5, PBS, and ψ) are required for encapsidation of a heterologous RNA;

iii) *cis* elements from the 5' UTR exhibit effects that can enhance heterologous vector titer without increasing RNA encapsidation; and iv) a packaging system competent for heterologous RNA encapsidation should minimally include the Rev/RRE system, all *cis* elements of the 5' UTR, and Gag polyprotein.

Mutation of *cis* elements in the canonical HIV-1 packaging signal mitigates encapsidation efficiency, but not Rev/RRE-mediated encapsidation. Data from the gain-of-function studies described above demonstrate that there are a number of components that must function together for a HIV-1 packaging system to achieve efficient encapsidation of a heterologous RNA into HIV-1 viral particles. In these studies the innate context of the HIV-1 *cis* elements was altered, therefore to bolster these findings mutagenesis and functional analysis were used to examine the contribution of HIV-1 *cis* elements from the 5' UTR, to RNA encapsidation. A previous study demonstrated that the HIV-1 Rev can enhance encapsidation of a HIV-1 vector into HIV-1 viral particles (19). In consonance with this study, results from three different methods, utilized here, show that HIV-1 Rev can indeed augment HIV-1 vector RNA encapsidation (Figs. 26 and 28). A standard HIV-1 vector system was used in which the luciferase gene was incorporated upstream of the endogenous CMV-GFP transgene cassette (Fig. 26A), therefore luciferase expression could be used as an indirect measure of full-length vector RNA in the cytoplasm. Rev enhanced titers of normalized vectors 40 fold (Fig. 26B), which were closely reflected in the RNA encapsidation levels as shown by northern blot analysis (Fig. 26C), and quantitated by the ratio of titer/cytoplasmic RNA exhibiting a 20 fold increase (Fig. 26D). The approximate 2 fold increase in cytoplasmic RNA revealed by northern blot was previously observed by

another lab using qRT-PCR (19). With the capacity to measure Rev-mediated encapsidation of an HIV-1 vector, mutagenesis could be used to examine the RNA packaging contribution of *cis* elements from the 5' UTR.

The influence of Rev on encapsidation of a HIV-1 vector with a deletion of the entire canonical packaging signal was initially investigated (Fig. 27A). However, it was quickly recognized that in the absence of the HIV-1 splice donor in stem-loop 2 (deleted with the rest of the canonical packaging signal) the HIV-1 Tat protein must be coexpressed to simply maintain expression of HIV-1 vector RNA from a CMV promoter. These results were in agreement with a previous study demonstrating that CMV-driven expression from a proviral clone is rendered Tat-dependent in the absence of the major splice donor (17). Therefore, experiments for figure 27 were executed in the presence of ectopically expressed HIV-1 Tat protein. Nevertheless, Rev augmented titers 6 fold (Fig. 27B), and correspondingly enhanced encapsidation as revealed by northern blot analysis (Fig. 27C). Indirect measuring of encapsidation by ratio of titer/cytoplasmic RNA (quantitated by densitometry from northern) exhibited an 11 fold increase in Rev-mediated encapsidation (Fig. 27D). In line with studies in chapter 2, and those described above, the Rev/RRE system can enhance encapsidation in the absence of *cis* elements in the canonical packaging signal. The requirement for Tat-dependent transcription of the vector RNA introduced an additional variable into the system that could be avoided by retaining stem-loop 2 with the splice donor sequence, as shown previously (117). HIV-1 vectors deleted of stem-loop 1 (contains dimerization domain) and the combination of stem-loop 1, 3, & 4 were generated, and assessed for encapsidation relative to a standard HIV-1 vector containing all *cis* elements (Fig. 28A). Deletion of stem-loop 1 resulted in a 5 fold decrease in titers (Fig. 28B) and 3 fold decrease in the

titer/luciferase ratio (Fig. 28C). More dramatic, however, was the composite deletion in stem-loops 1, 3, & 4 resulting in a 18 fold decrease in both titer (Fig. 28B) and titer/luciferase ratio (Fig. 28C). The RNA encapsidation efficiency exhibited a correlative trend in that deletion of multiple *cis* elements (stem-loops 1,3, & 4) in the canonical packaging signal resulted in a 4 fold decrease in the encapsidation efficiency in the presence of the Rev/RRE system (Fig. 28D). Despite deletion of *cis* elements in the canonical packaging signal yielding reduced encapsidation efficiency, the Rev/RRE system retained the capacity to augment RNA encapsidation for each vector RNA. These results bolster previous findings in that the Rev/RRE system can positively influence RNA encapsidation in the absence of canonical *cis* packaging elements. These results also confirm that a complete HIV-1 packaging system requires the Rev/RRE system, *cis* elements in the 5' UTR, and Gag polyprotein to achieve efficient and specific encapsidation.

The HIV-1 Rev/RRE and 5' UTR *cis* elements determine specificity for encapsidating RNA into HIV-1 viral particles. Manipulating the HIV-1 packaging system, described above, to efficiently encapsidate a foreign RNA into HIV-1 viral particles may be a general biological phenomenon that can be exploited for RNA encapsidation into retroviral particles that are not derived from HIV-1. The heterologous MLV RNA vector system was used to examine if the HIV-1 Rev/RRE and 5' UTR *cis* elements can mediate packaging of RNA into MLV viral particles, thereby resulting in a loss of specificity for HIV-1 viral particles. Retention of the MLV packaging signal bestows dual functionality onto the MLV/HIV RRE+RU5PS chimeric vector RNA to also allow for packaging into MLV derived viral particles (Fig. 29A). Accordingly, this vector was used to examine if the HIV-1 Rev/RRE

and 5' UTR *cis* elements alter the encapsidation efficiency into MLV viral particles. Direct comparison of the chimeric vector to the basic MLV vector revealed that there was little impact of the Rev/RRE system on normalized titers or luciferase levels (Fig. 29C and D). In fact, transduction with equivalent units of MLV reverse transcriptase resulted in a reduced number of transduced cells, compared to the basic MLV vector (Fig. 29B). Moreover, the titers were slightly lower than those of the basic MLV vector, resulting in decreased levels after normalization to luciferase (Fig. 29E). In contrast to HIV-1 viral particles, these data indicated that the HIV-1 Rev/RRE and 5' UTR *cis* elements do not influence the packaging of RNA into MLV viral particles.

Investigation of the encapsidation efficiency into MLV viral particles exposed a picture similar to that obtained with the titer/luciferase assays. There was no effect of the Rev/RRE and 5' UTR *cis* elements on levels of vector RNA in MLV viral particles, or in the producer cell cytoplasm (Fig. 30A and B). Consequently, these HIV-1 components also had no effect on RNA encapsidation into MLV viral particles (Fig. 30C). These results imply that the Rev/RRE system and 5' UTR *cis* elements confer specificity onto the heterologous MLV vector RNA for encapsidation into HIV-1 viral particles, but provide no advantage for encapsidation into MLV viral particles. Using a single RNA system with different packaging specificities we were able to demonstrate that HIV-1 and MLV commandeer distinct mechanisms to select vector RNAs from the milieu of host cell RNAs to promote RNA encapsidation into nascent viral particles.

The promiscuous packaging of host cell RNAs into HIV-1 viral particles (188) allows for particles to be generated in the absence of a packaging competent viral genomic RNA (28). Therefore, there is a need for the virus to have a packaging system that can

preferentially package the viruses own genome to maintain viral fitness. Since HIV-1 viral particles exhibit a limited capacity for the amount of RNA packaged into viral particles, it could be anticipated that a competent packaging system could confer enhanced packaging of a specific vector/viral RNA while concomitantly excluding the packaging of nonspecific cellular RNA. HIV-1 derived lentiviral vectors are commonly generated in 293T human embryonic kidney cells. 293T cells overexpress the SV40 large T antigen, which is also a well recognized tumorigenic protein. The specificity of packaging a HIV-1 derived vector was compared to that of the large T antigen. HIV-1 vectors were generated in 293T producer cells in the absence and presence of HIV-1 Rev protein. Vector RNA was harvested from equivalent levels of viral particles and assessed by qRT-PCR. Large T antigen RNA was readily detectable as a packaged RNA in viral particles harvested from the media (Fig. 31A), and in concentrated viral particles (Fig. 31C). Packaging of large T antigen RNA exhibited an inverse relationship with packaging of vector RNA (Figs. 31A and B). In the presence of Rev there was a 17 fold decrease in large T antigen RNA (Fig. 31A), whereas HIV-1 viral vector RNA was dramatically increased (Fig. 31B). These data demonstrate that the specificity conferred upon the vector RNA by the Rev/RRE system can mitigate the nonspecific packaging of producer cell RNAs. Nonetheless, this does not eliminate the possibility of transferring large T antigen RNA to naïve host cells. Eliminating the potential to package large T antigen RNA may require changing the vector producer cell line. The SODk0 cell line, developed in our lab (44), is derived from 293 cells (i.e. no large T antigen). HIV-1 vectors generated in this cell line do not have large T antigen (Fig. 31C), albeit titers may be diminished up to 20 fold. To ascertain if packaged large T antigen RNA can be reverse transcribed following transduction total DNA was isolated from SODk0 cells

transduced in the absence or presence of the reverse transcriptase inhibitor, etravirine (Fig. 31D). Large T antigen cDNA was detected only in the absence of etravirine, an indication that the large T antigen RNA is reverse transcribed into cDNA. Therefore, the potential exists for retroviral vectors produced in 293T cells to transfer large T antigen to host cells targeted for gene therapy protocols.

Discussion

The objective of these studies was to understand how the well characterized *cis* elements in the 5' UTR, and the Rev/RRE system, function in a concerted manner to achieve efficient and specific RNA encapsidation into HIV-1 viral particles. Most importantly, the HIV-1 Rev/RRE system was required for *cis* elements in the 5' UTR to mediate efficient encapsidation into HIV-1 viral particles (Figs 20-24). These studies were bolstered by the finding that the canonical packaging signal, in the more natural setting of a HIV-1 vector RNA, was necessary to retain efficient encapsidation, as might be expected; however, the Rev/RRE system preserved the capacity to enhance encapsidation in the absence of the canonical packaging signal (Figs. 26-28). Furthermore, prior mutagenesis studies have implicated 5' UTR *cis* determinants adjacent to the canonical packaging signal as important for efficient RNA encapsidation through loss of function analysis (37, 38, 81, 139). Data presented here expand this view by demonstrating that the 5' UTR *cis* elements are not separable to achieve efficient encapsidation of heterologous RNAs (Fig. 25); quite possibly indicating a requirement for intramolecular interactions between *cis* elements in the R, U5, and PBS, with those in the canonical packaging signal, as previously indicated (50, 214). These studies are an expansion of the conclusion reached in chapter 2 and coalesce previous

work in the field demonstrating that a complete packaging system is comprised of the Rev/RRE, *cis* elements in the 5' UTR, and Gag polyprotein.

Lentiviruses may require a sophisticated encapsidation mechanism to ensure that full-length viral RNAs transcribed in the nucleus can effectively be packaged into nascent viral particles at the plasma membrane. Conceivably, the Rev/RRE may impact the cytoplasmic distribution of RNA without direct involvement in packaging RNA into viral particles at the plasma membrane, a view consistent with the inability to detect HIV-1 Rev in viral particles (71). Transferring viral genomic RNA to subcellular sites where the Gag polyprotein is available for interaction with the canonical packaging signal would allow for subsequent encapsidation by Gag, independent of Rev. As described in chapter 2 Rev is able to recruit a number of cellular proteins to the RNA, some of which can alter the subcellular location of Rev (47, 56, 80, 147, 180, 189, 196, 222, 223). Additionally, the Rev/RRE system may alter the conformation of the 5' UTR creating a context that is more receptive to an interaction with HIV-1 Gag, and subsequent packaging. Recent *in vitro* evidence indicates that HIV-1 Rev can influence translation in a concentration dependent manner that does not rely upon the RRE, but rather an interaction between Rev and a *cis* determinant in stem-loop 1 of the 5' UTR (70). At moderate concentrations Rev enhanced translation, but at high concentrations translation was inhibited (70). The same interaction was also implicated in RNA encapsidation (67), therefore it is plausible that Rev may promote RNA packaging at high concentrations, possibly acting as a "switch" between translation and encapsidation. Nonetheless, experiments supporting such a mechanism are still required. These studies indicate that a Rev mechanism may also depend upon recognition of *cis* determinants in the canonical packaging signal.

Understanding that the 5' UTR is central to the encapsidation mechanism studies were executed to examine the contribution of various stem-loops in the canonical packaging signal to encapsidation of a HIV-1 vector RNA into HIV-1 viral particles. In agreement with studies described in chapter 2, the Rev/RRE system was able to mediate encapsidation upon deletion of the entire canonical packaging signal (stem-loops 1-4) from an HIV-1 derived vector (Fig. 27). However, as previously demonstrated (17) vector RNA expression was rendered Tat-dependent in the absence of the major splice donor; therefore individual stem-loops were deleted, while maintaining stem-loop 2 with the splice donor. Deletions of stem-loop 1 and stem-loops 1, 3, 4 resulted in decreased RNA efficiency of RNA encapsidation compared to a fully competent packaging signal (Fig. 28). This is in agreement with a previous report demonstrating that deletion of various stem-loops in ψ impairs RNA packaging (89, 117), but does not eliminate packaging and subsequent transduction. Accordingly, mutations in the packaging signal can alter specificity, favoring increased packaging of non-specific RNAs into HIV-1 viral particles (89). Furthermore, as demonstrated here mutations in the stem-loops did not eliminate the capacity of the Rev/RRE system to augment encapsidation of each vector RNA with a mutation in the canonical packaging signal. Discerning how the Rev/RRE system influences specific and efficient recognition of *cis* elements in the 5' UTR by the Gag polyprotein will be an important focus of future studies.

The requirement for the HIV-1 Rev/RRE system in the encapsidation mechanism implies that the Rev/RRE may confer specificity onto HIV-1 RNA during the initial steps of the mechanism when Rev interacts with RNA in the nucleus. Such a mechanism might ensure early selection of viral RNA from the milieu of host cell RNAs, concomitant with

transcription in the nucleus. Our data support a role for the Rev/RRE and 5' UTR *cis* elements in conferring specificity of RNA packaged into HIV-1 viral particles (Figs. 24 & 29-31). The HIV-1 Rev/RRE did not confer an encapsidation advantage of the heterologous RNA into MLV viral particles (Figs. 29 & 30); consistent with the notion that HIV-1 and MLV utilize distinct mechanisms to encapsidate viral RNA (53). In the absence of the HIV-1 Rev/RRE, MLV vector RNA exhibited no added specificity upon packaging into HIV-1 viral particles, in agreement with a study showing that a MLV vector RNA was not enriched in HIV-1 viral particles relative to cellular mRNAs (188). Nonspecific (yet measurable) packaging of MLV RNA raise the concern that stable HIV-1 packaging cell lines (i.e. for lentiviral vector production), generated by introducing HIV-1 *gag* and *pol* genes with MLV vectors (92, 204), have the potential to package, transfer, reverse transcribe, and recombine HIV-1 *gag* and *pol* genes in recipient cells. Also of concern regarding the use of lentiviral vectors in gene therapy protocols is the potential for transfer of nonspecifically packaged RNAs derived from vector producer cells.

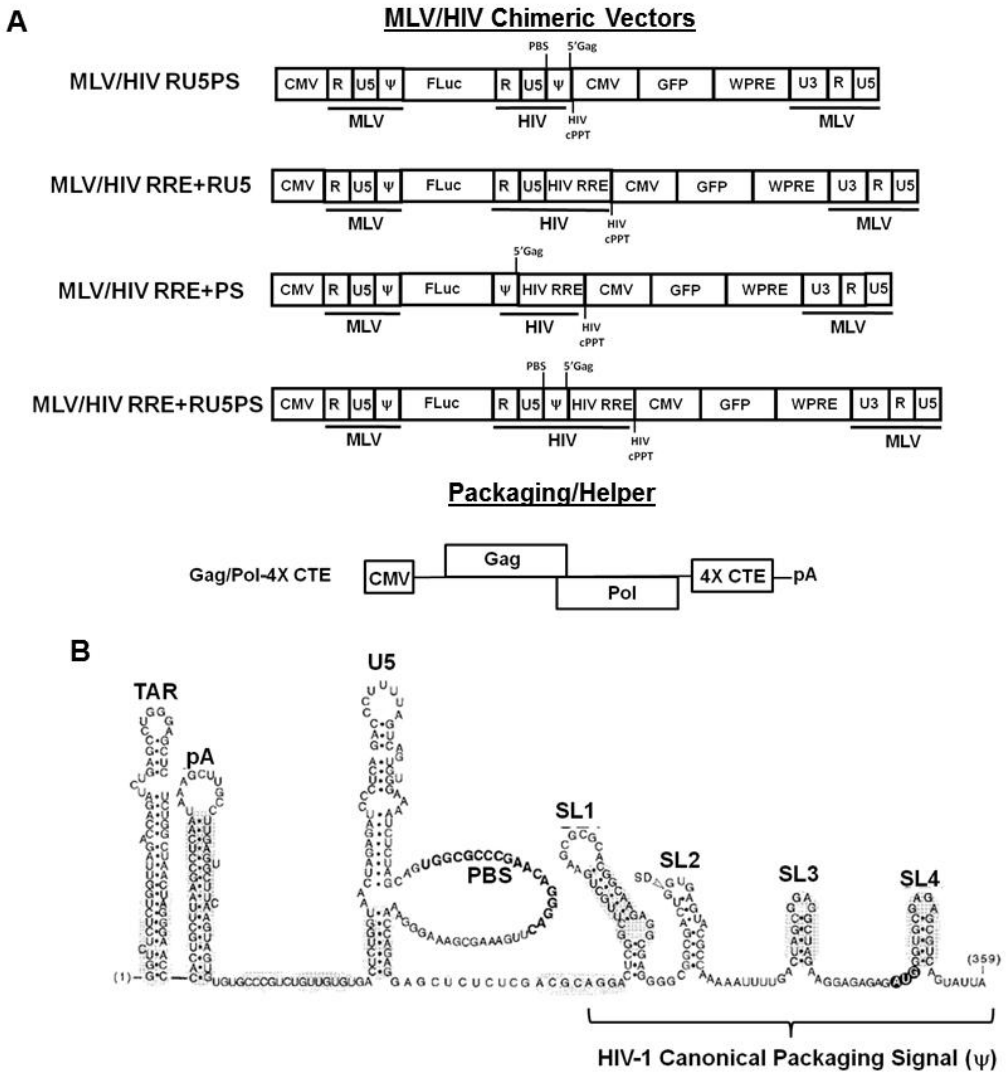
Nonspecific packaging of cellular RNAs during generation of nascent HIV-1 viral particles has been well documented (152, 162, 188). Importantly, packaged cellular RNAs have been shown to be reverse transcribed, and recombine with the packaged viral RNA during reverse transcription in a transduced host cell; thereby, potentially being integrated and expressed in a naïve host cell (78, 123, 151). With regard to retroviral vector production, 293T cells were identified to be most amenable for this process through ectopic expression of vector particle components (chapter 1, Fig. 5). Most notably 293T cells overexpress the large T antigen, which is well recognized to transform cells upon overexpression. Data presented here demonstrate that large T antigen RNA is readily packaged into HIV-1 derived viral

particles and can be reverse transcribed in naïve host cells (Fig. 31); however, this concern can be eased by utilizing cells that do not overexpress large T antigen (Fig. 31). The potential for a retrovirus or retroviral vector in this case, to acquire a host cell oncogene, and transfer that oncogene to naïve host cells is not an unexpected possibility. The well characterized avian Rous sarcoma virus is an oncoretrovirus that has acquired the cellular src-oncogene with full capacity to transfer the functional oncogene to naïve host cells (202), and induce tumor formation. In addition to the potential for transferring an RNA encoding a tumorigenic protein, these data demonstrate that the specificity conferred upon the vector RNA by the Rev/RRE system can mitigate the nonspecific packaging of producer cell RNAs (i.e. Rev increases packaging of HIV-1 vector RNA while concomitantly reducing nonspecific large T antigen RNA).

Although our system revealed an essential role for the Rev/RRE system in efficient and specific RNA encapsidation into HIV-1 viral particles, a discrepancy was observed between the efficiency of RNA encapsidation (Fig. 24) and p24 normalized titers (Fig. 21) for heterologous vector RNAs harboring the entire contingent of *cis* elements from the 5' UTR. The larger increase in titers indicates that *cis* elements from the 5' UTR may have pleiotropic effects that can impact different stages of the viral life cycle (i.e. reverse transcription during transduction). Inclusion of the HIV-1 primer binding site (PBS) and flanking 5' UTR sequences might accommodate more efficient reverse transcription and transduction of the heterologous RNA, yielding higher titer in the form of GFP positive cells. Efficient reverse transcription from the HIV-1 PBS is accomplished by the specific packaging of $tRNA^{Lys}$ primers into HIV-1 viral particles (1). Furthermore, the $tRNA^{Pro}$ primers may also promote reverse transcription at a low efficiency from the MLV PBS

(normally primed by $tRNA^{Pro}$) in the heterologous MLV vectors that lack a HIV-1 PBS; a rationalization consistent with a study showing that, although impaired, HIV-1 replication was retained if the HIV-1 PBS was altered to that of MLV (51).

Using a heterologous MLV RNA system we were able to isolate the encapsidation effects of different HIV-1 *cis* and *trans* components. Most importantly, however, these studies revealed the concerted effects of multiple HIV-1 components through gain-of-function studies. Conventional loss-of-function studies have implicated several of the aforementioned HIV-1 *cis* and *trans* components in the encapsidation mechanism, but do not reveal the interdependence of these components. These data demonstrate that the HIV-1 Rev/RRE system is essential for *cis* elements in the 5' UTR (including the canonical packaging signal) to mediate efficient and specific encapsidation of a heterologous RNA into HIV-1 viral particles. Therefore, in addition to its traditional role in nuclear export, the Rev/RRE system may have a critical role in making *cis* elements in the 5' UTR of HIV-1 RNA more amenable to RNA-RNA and RNA-protein interactions in the cytoplasm, which support subsequent RNA encapsidation. Nonetheless, the combined effects of the Rev/RRE system and 5' UTR *cis* elements are not limited to encapsidation since these components can synergize to yield transduction efficiencies that approach those of standard HIV-1 vectors. Heterologous RNAs packaged into HIV-1 viral particles exhibit unique transduction properties that could be beneficial for gene therapy protocols, and provide a model system to study the molecular virology of retroviruses encountered at different stages of early infection. Studies investigating unique transduction properties associated with heterologously packaged RNAs are described in chapter 4.



Adapted by Permission from American Society for Microbiology. Clever, J.L., Miranda Jr., D., & Parslow, T.G. RNA Structure and Packaging Signals in the 5' Leader Region of the Human Immunodeficiency Virus Genome. *Journal of Virology.* 2002. 76(23). 12381-12387.

Figure 20. Incorporation of HIV-1 5'UTR cis elements into MLV/HIV chimeric vectors. A. Full-length MLV/HIV chimeric vector RNAs are expressed from a CMV (cytomegalovirus) promoter in transfected 293T cells. MLV and HIV *cis* elements can be distinguished by black underscore. Chimeric vector names are represented as MLV/HIV followed by corresponding HIV *cis* elements incorporated: RRE (Rev Response Element), R (repeat), U5 (unique region 5), PS (packaging signal comprised of ψ [canonical packaging signal and into 5' Gag region]), cPPT (central polypurine tract), PBS (primer binding site). Also incorporated are the WPRE (woodchuck hepatitis virus post-transcriptional regulatory element), FLuc (firefly luciferase gene), and GFP (green fluorescent protein gene). HIV-1 Gag-Pol 4X CTE helper construct was used to express structural and enzymatic proteins to generate viral particles independent of HIV-1 Rev protein. **B.** RNA *cis* elements in the HIV-1 5' UTR. TAR and pA comprise most of the R region. Further details are described in figure 7.

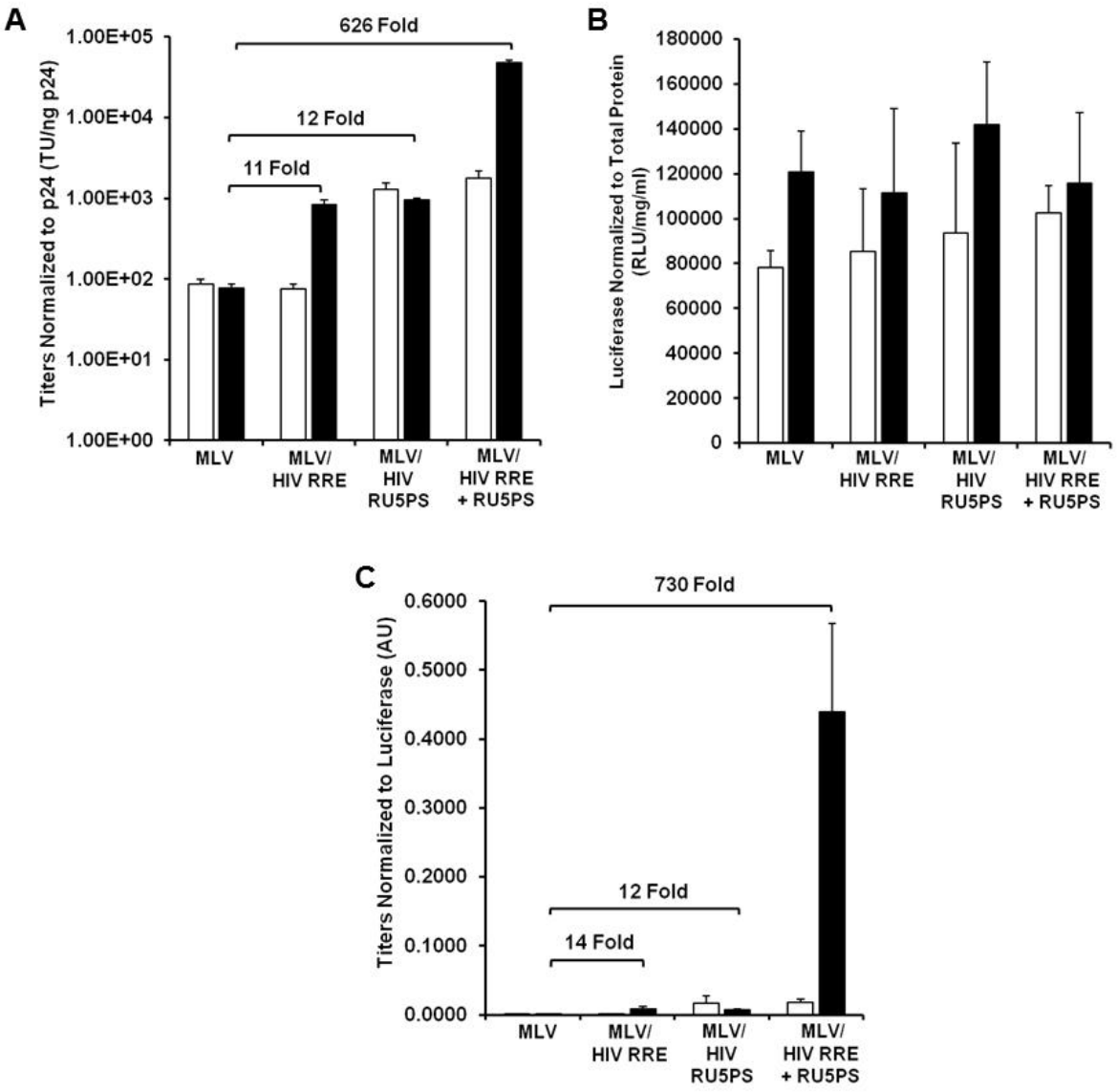


Figure 21. HIV-1 *cis* elements in the 5'UTR and Rev/RRE system augment vector titers, but do not influence cytoplasmic luciferase levels. A. Vector titers normalized to p24 are shown in the absence (white bars) and presence (black bars) of Rev. **B.** Luciferase levels normalized to total protein are shown for each vector. **C.** Titers expressed as a ratio to luciferase are shown as arbitrary units (AU). In all graphs the influence of adding HIV-1 *cis* elements to the MLV vector is indicated by fold increases in the presence of Rev relative to the standard MLV vector. Error for all bar graphs is expressed as ±S.D. All experiments were performed in triplicate.

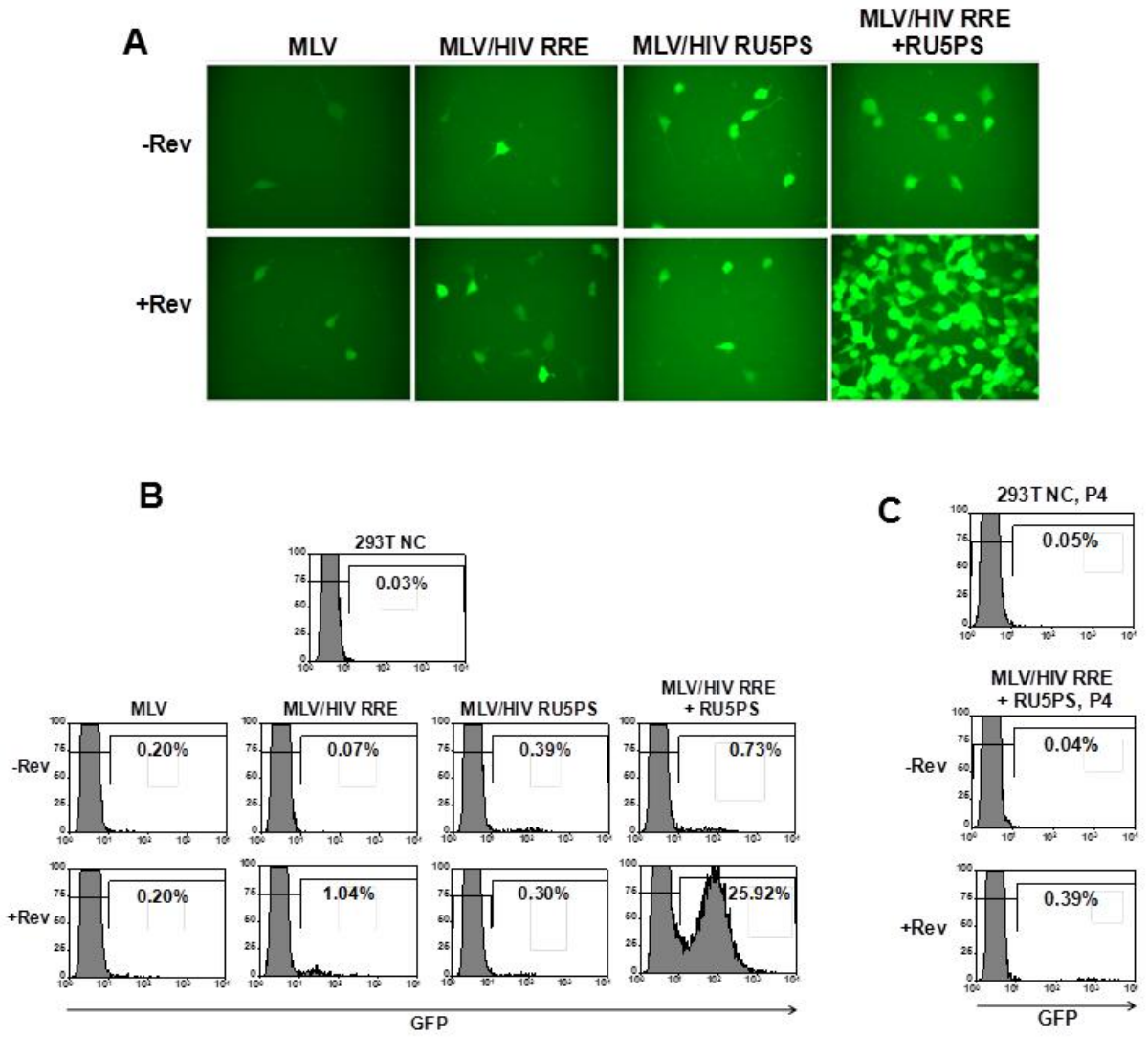


Figure 22. Transduction of 293T cells with chimeric MLV/HIV vectors packaged into HIV-1 viral particles. A and B. 293T cells were transduced with equivalent amounts of p24 capsid protein (50 ng), as determined for each of the indicated chimeric vectors. The influence of the HIV-1 Rev/RRE system, and 5' UTR *cis* elements, on transduction was assessed by fluorescence microscopy **(A)** and FACscan analysis **(B)** at 7 days post-transduction. **C.** The capacity of the MLV/HIV RRE+RU5PS vector to be stably maintained after 4 cell passages was examined by FACscan analysis. The percent GFP positive cells are indicated for each FACscan and 293T negative control (NC) cells are shown.

.

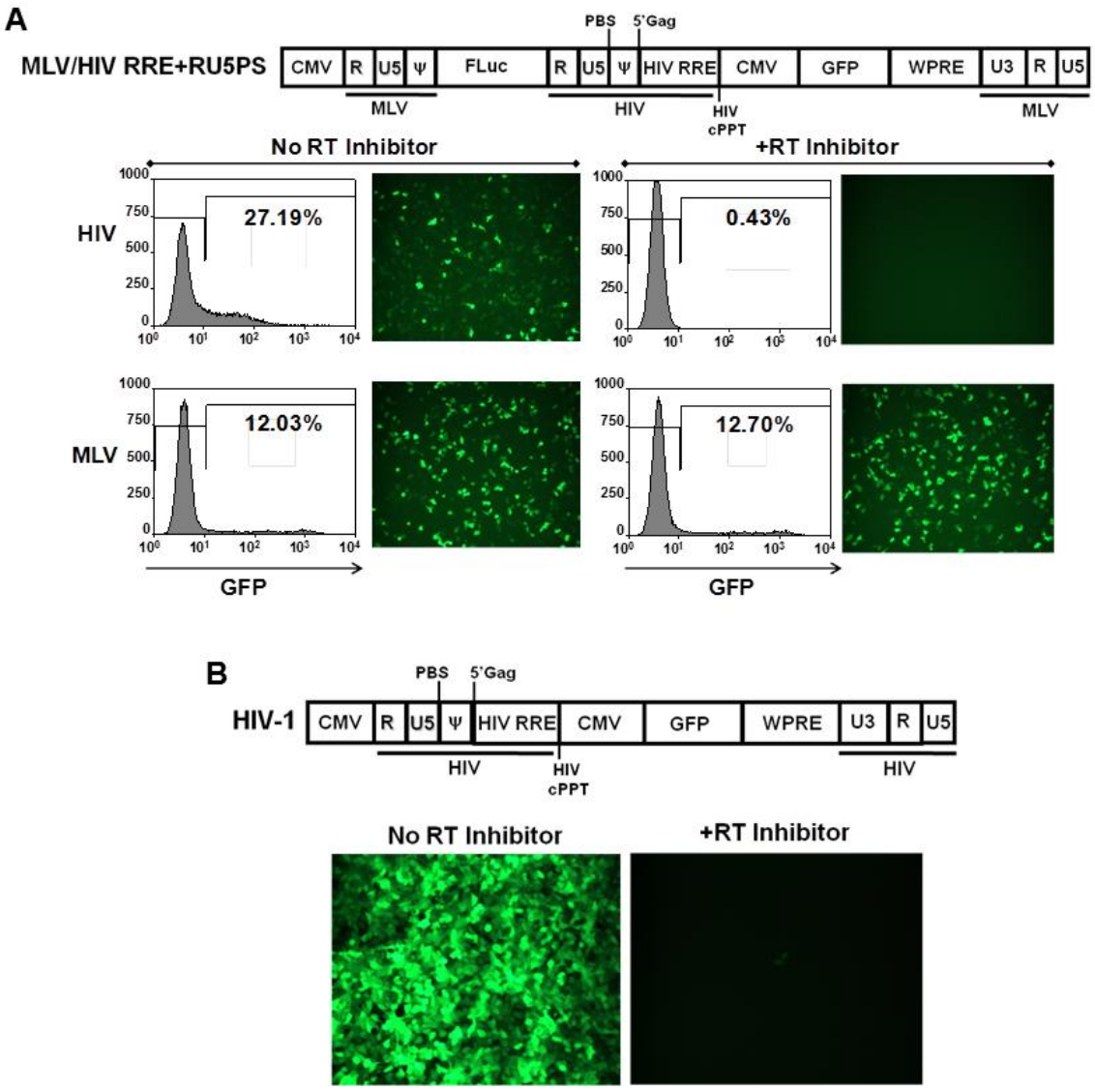


Figure 23. Transduction of chimeric MLV/HIV RRE+RU5PS and HIV-1 vectors is dependent on reverse transcription. 293T cells were transduced in the absence (No RT Inhibitor), or presence (+RT Inhibitor), of the HIV-1 specific non-nucleoside reverse transcriptase inhibitor, etravirine (100 nM). **A.** 293T cells transduced with MLV/HIV RRE + RU5PS were assessed by fluorescence microscopy and FACscan analysis. **B.** 293T cells transduced with a HIV-1 vector were assessed by fluorescence microscopy.

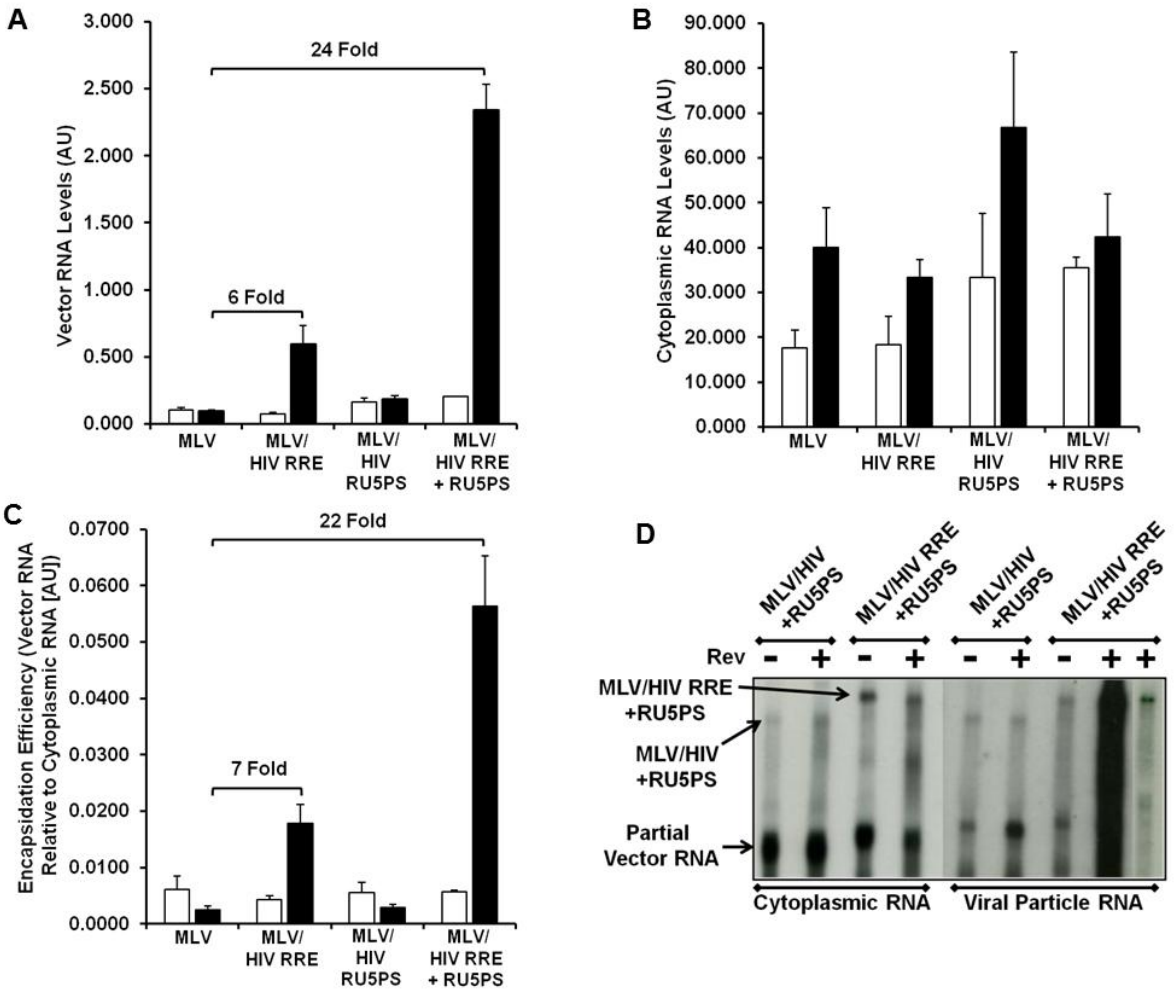


Figure 24. HIV-1 *cis* elements in the 5'UTR and Rev/RRE system cooperatively enhance RNA encapsidation into HIV-1 viral particles. A. Vector RNA was measured by qRT-PCR and expressed in arbitrary units (AU). RNA levels for all graphs are shown in the absence (white bars) and presence (black bars) of Rev. The influence of adding HIV-1 *cis* elements to the MLV vector is indicated by fold increases in the presence of Rev relative to the standard MLV vector. **B.** Cytoplasmic RNA was isolated from vector producer 293T cells at the time of vector harvesting. Relative RNA levels were obtained and recorded as done for vector RNA in part A. **C.** Efficiency of encapsidating RNA into HIV-1 viral particles is expressed as a ratio of vector RNA in viral particles to cytoplasmic RNA available for encapsidation. Relative levels are expressed like vector RNA in part A. **D.** Northern blot analysis of cytoplasmic and vector RNA isolated from MLV/HIV RU5PS and MLV/HIV RRE+RU5PS in the absence (-) and presence (+) of Rev. Vector length RNA species were detected with a probe to a region in the 5' end of the vector, as well as an additional RNA species (labeled 'partial vector RNA'). Cytoplasmic and vector RNAs are shown at different exposures of the same blot. Last lane (far right) is a shorter exposure of adjacent left lane. Error for all bar graphs is expressed as ±S.D. All experiments were performed in triplicate.

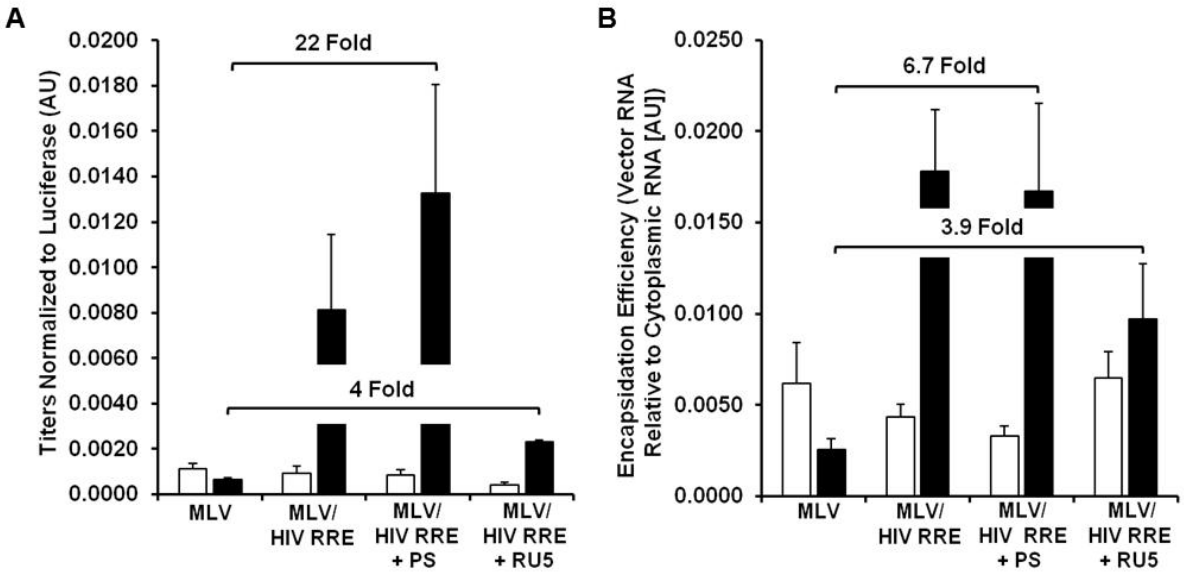


Figure 25. *Cis* elements in the HIV-1 5' UTR are not separable. A. Titers expressed as a ratio to luciferase are shown in the absence (white bars) and presence (black bars) of Rev, and expressed as arbitrary units (AU). **B.** Efficiency of encapsidating RNA into HIV-1 viral particles is expressed as a ratio of vector RNA in viral particles to cytoplasmic RNA available for encapsidation. Encapsidation efficiency is expressed as arbitrary units (AU). In all graphs the influence of adding HIV-1 *cis* elements to the MLV vector is indicated by fold increases in the presence of Rev relative to the standard MLV vector. Error for all bar graphs is expressed as ±S.D. All experiments were performed in triplicate.

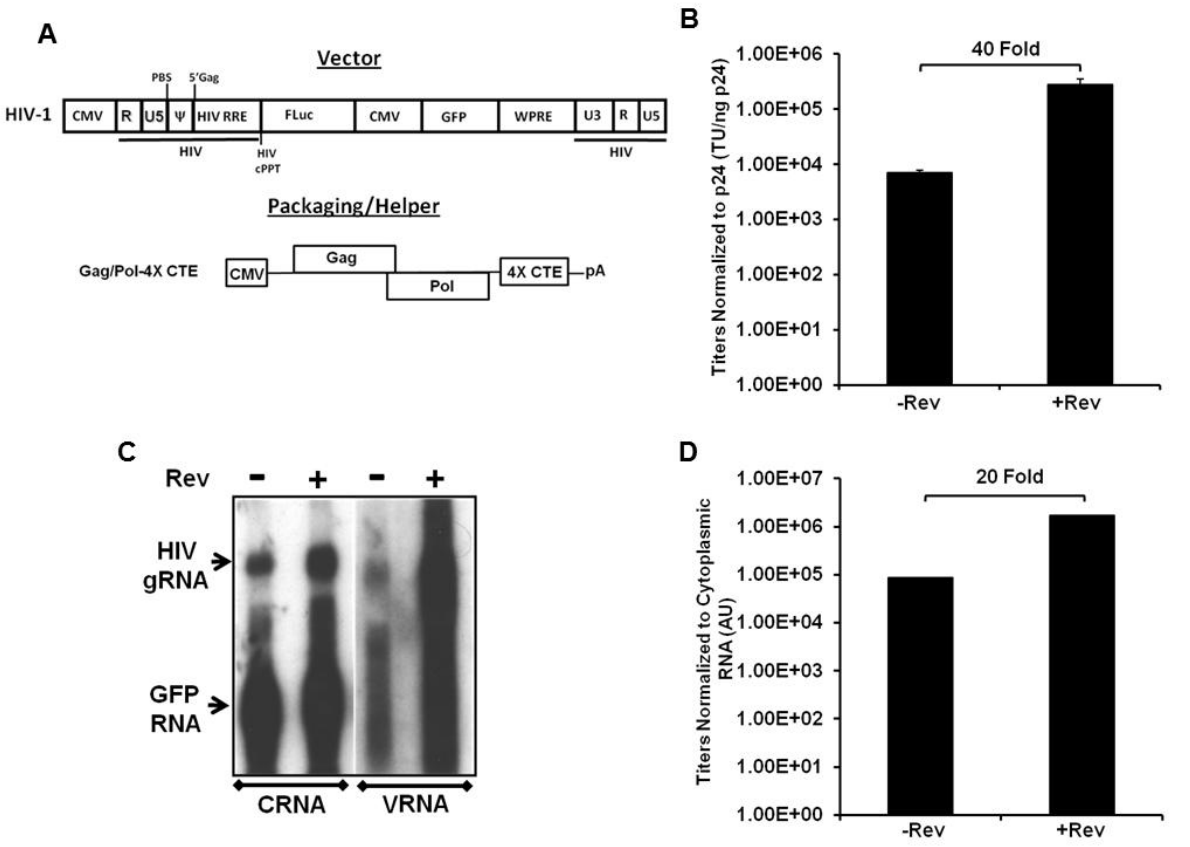


Figure 26. Rev/RRE system augments encapsidation of HIV-1 vector RNA into HIV-1 viral particles. A. Viral particles were produced in 293T cells packaging a standard HIV-1 vector into HIV-1 viral particles generated from the Gag/Pol 4X CTE packaging construct. **B.** Vector titers normalized to p24 are shown in the absence and presence of Rev. **C.** Northern blot analysis of cytoplasmic (CRNA) and vector RNA (VRNA) isolated from HIV-1 vector in the absence (-) and presence (+) of Rev. Vector length RNA species were detected with a probe to the GFP in the 3' end of the vector, as well as an additional RNA species (labeled 'GFP RNA'). **D.** Indirect encapsidation efficiency is represented as a ratio of titers to cytoplasmic RNA, which was quantitated from the northern blot. The ratio is expressed in arbitrary units (AU). Error (were indicated) for bar graphs is expressed as ±S.D.

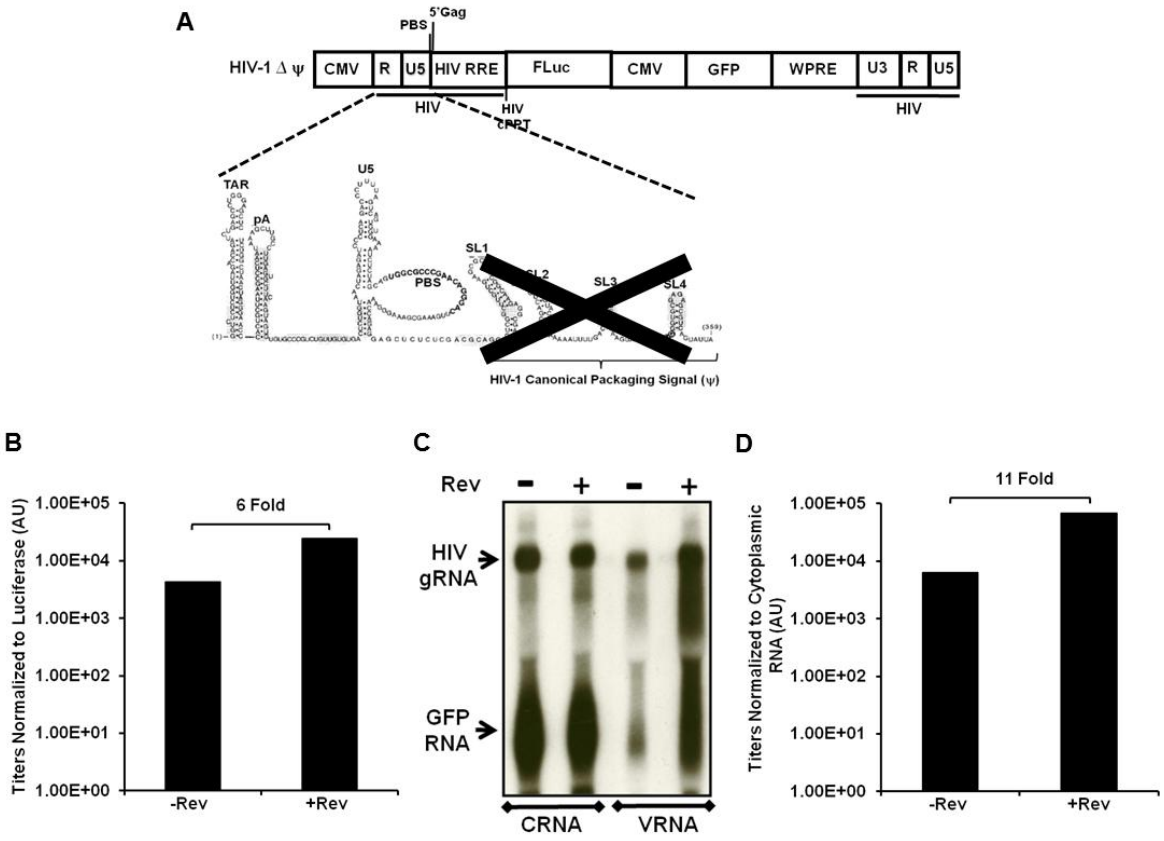


Figure 27. The HIV-1 Rev/RRE system augments encapsidation of a HIV-1 derived vector lacking the entire canonical packaging signal. A. HIV-1 Δ ψ is a standard HIV-1 vector deleted of the entire canonical packaging signal (stem-loops 1-4). **B.** Titers expressed as a ratio to luciferase are shown in the absence and presence of Rev, and expressed as arbitrary units (AU). **C.** Northern blot analysis of cytoplasmic (CRNA) and vector RNA (VRNA) isolated from HIV-1 vector in the absence (-) and presence (+) of Rev. Vector length RNA species were detected with a probe to the GFP in the 3' end of the vector, as well as an additional RNA species (labeled 'GFP RNA'). **D.** Indirect encapsidation efficiency is represented as a ratio of titers to cytoplasmic RNA, which was quantitated from the northern blot. The ratio is expressed in arbitrary units (AU).

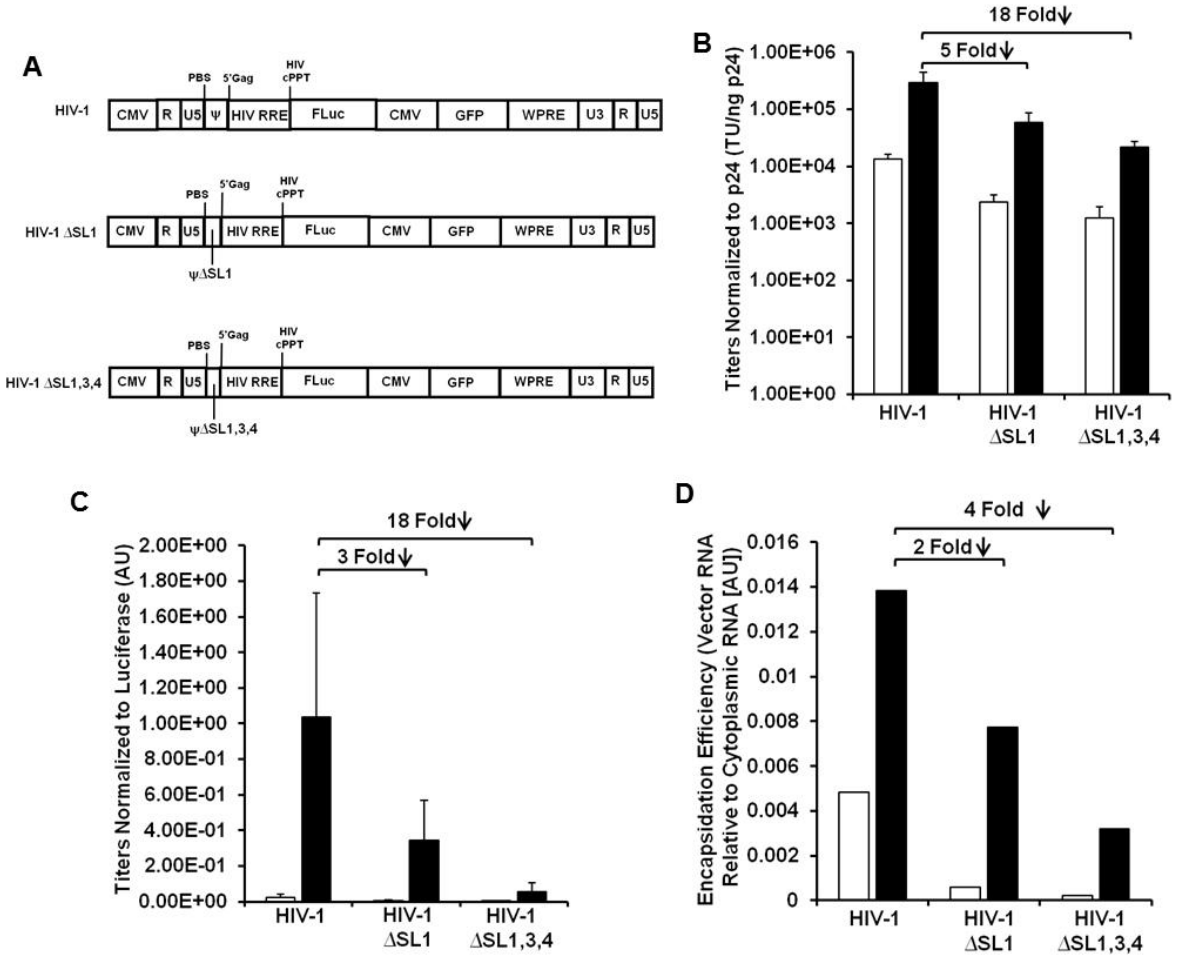


Figure 28. Stem-loop deletions in the canonical HIV-1 packaging signal exhibit reduced encapsidation efficiency. A. HIV-1 derived vectors with no deletions (HIV-1), deletion of stem-loop 1 in ψ (HIV-1 ΔSL1), and deletion of stem-loops 1, 3, 4 in ψ (HIV-1 ΔSL 1, 3, 4). **B.** Vector titers normalized to p24 are shown in the absence and presence of Rev. **C.** Titers expressed as a ratio to luciferase are shown in the absence and presence of Rev, and expressed as arbitrary units (AU). **D.** Efficiency of encapsidating RNA into HIV-1 viral particles is expressed as a ratio of vector RNA in viral particles to cytoplasmic RNA available for encapsidation. Encapsidation efficiency was determined by qRT-PCR and is expressed as arbitrary units (AU). In all graphs the influence of deleting HIV-1 stem-loops from the canonical packaging signal of the HIV-1 vector is indicated by fold increases in the presence of Rev relative to the standard HIV-1 vector. Error (were indicated) for bar graphs is expressed as ±S.D.

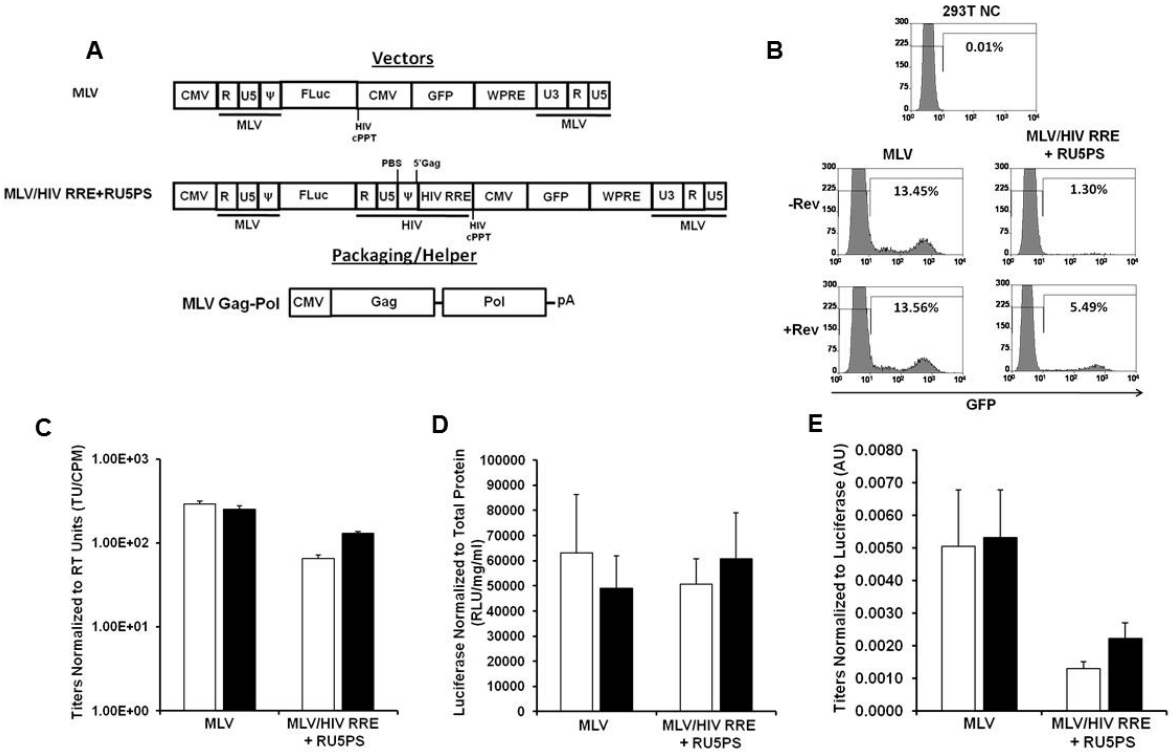


Figure 29. HIV-1 Rev/RRE and *cis* elements in the 5'UTR do not influence vector titers after packaging into MLV viral particles. A. The standard MLV vector and the MLV/HIV RRE + RU5PS chimeric vector RNAs (containing all the HIV-1 5' UTR *cis* elements), were assessed for encapsidation into MLV derived viral particles produced in 293T cells with a MLV Gag-Pol helper construct. **B.** 293T cells were transduced with equivalent amounts of RT units ($6x10^5$ CPM), as determined for each of the indicated chimeric vectors. The influence of the HIV-1 Rev/RRE system, and 5' UTR *cis* elements, on transduction was assessed by FACscan analysis at 7 days post-transduction. The percent GFP positive cells are indicated for each FACscan. **C.** Titers of MLV/HIV chimeric vectors were obtained by scoring for GFP positive cells following transduction of 293T cells. Titers are expressed as transducing units (TU) normalized to the amount of RT units (counts per minute [CPM]). **D.** Normalized luciferase levels were determined in transfected 293T producer cells. Luciferase levels were normalized to total cell protein. **E.** Titers (part A) expressed as a ratio to levels of luciferase (part B) shown in arbitrary units (AU). All experiments were executed in the absence (white bars) and presence (black bars) of Rev. Error for all bar graphs is expressed as ±S.D.

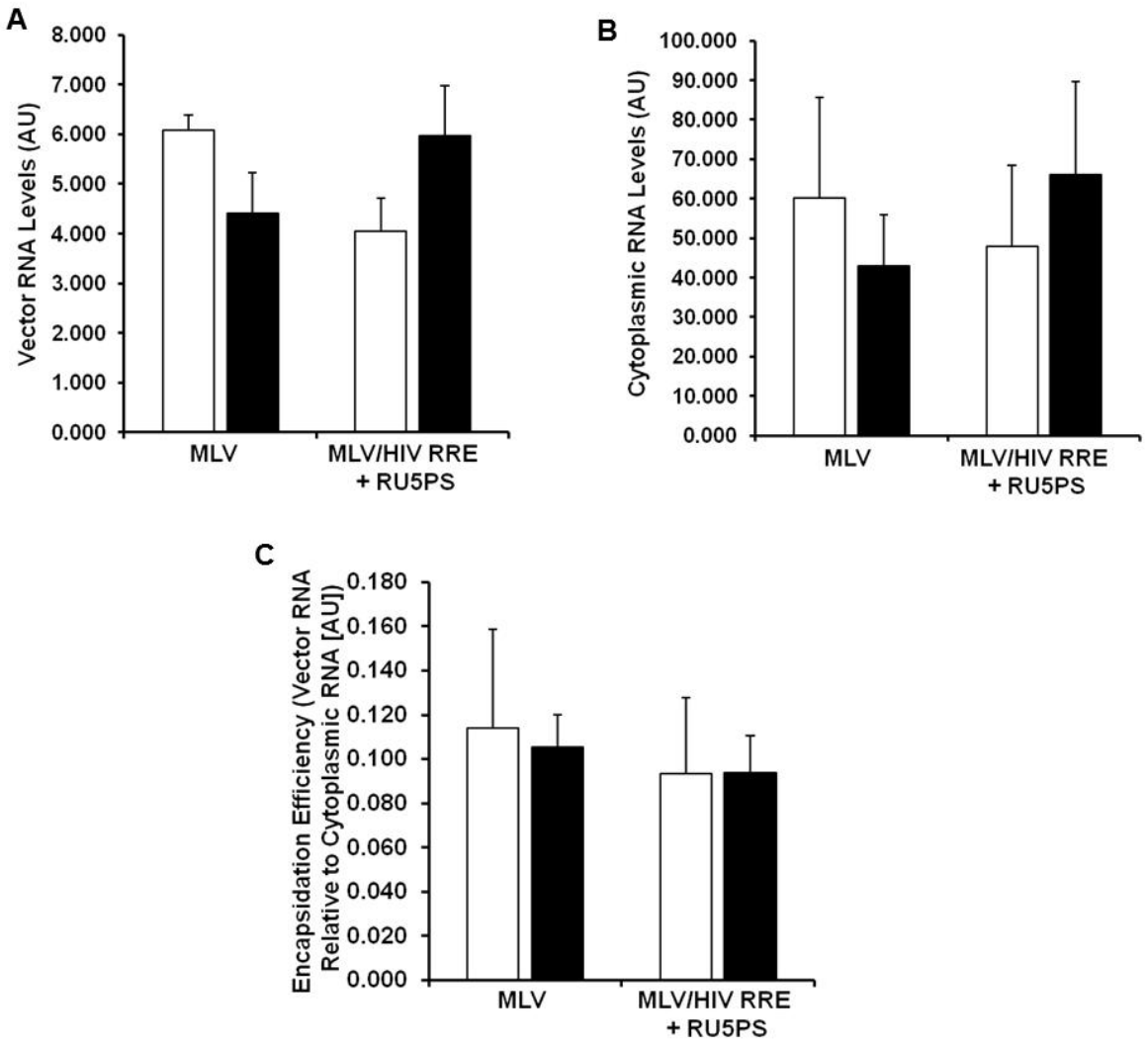


Figure 30. HIV-1 Rev/RRE and *cis* elements in the 5' UTR do not augment RNA encapsidation into MLV viral particles. A. Vector RNA packaged into MLV derived viral particles was isolated from equivalent amounts of RT units in the media of 293T producer cells. RNA levels were measured by qRT-PCR and are expressed as arbitrary units (AU). RNA levels are shown in the absence (white bars) and presence (black bars) of Rev. **B.** Cytoplasmic RNA was isolated from vector producer cells coincident with harvesting vector particles. Relative levels are expressed similar to vector RNA in part A. **C.** Efficiency of encapsidating RNA into MLV viral particles is expressed as a ratio of vector RNA in viral particles to cytoplasmic RNA available for encapsidation. Error for all bar graphs is expressed as ±S.D.

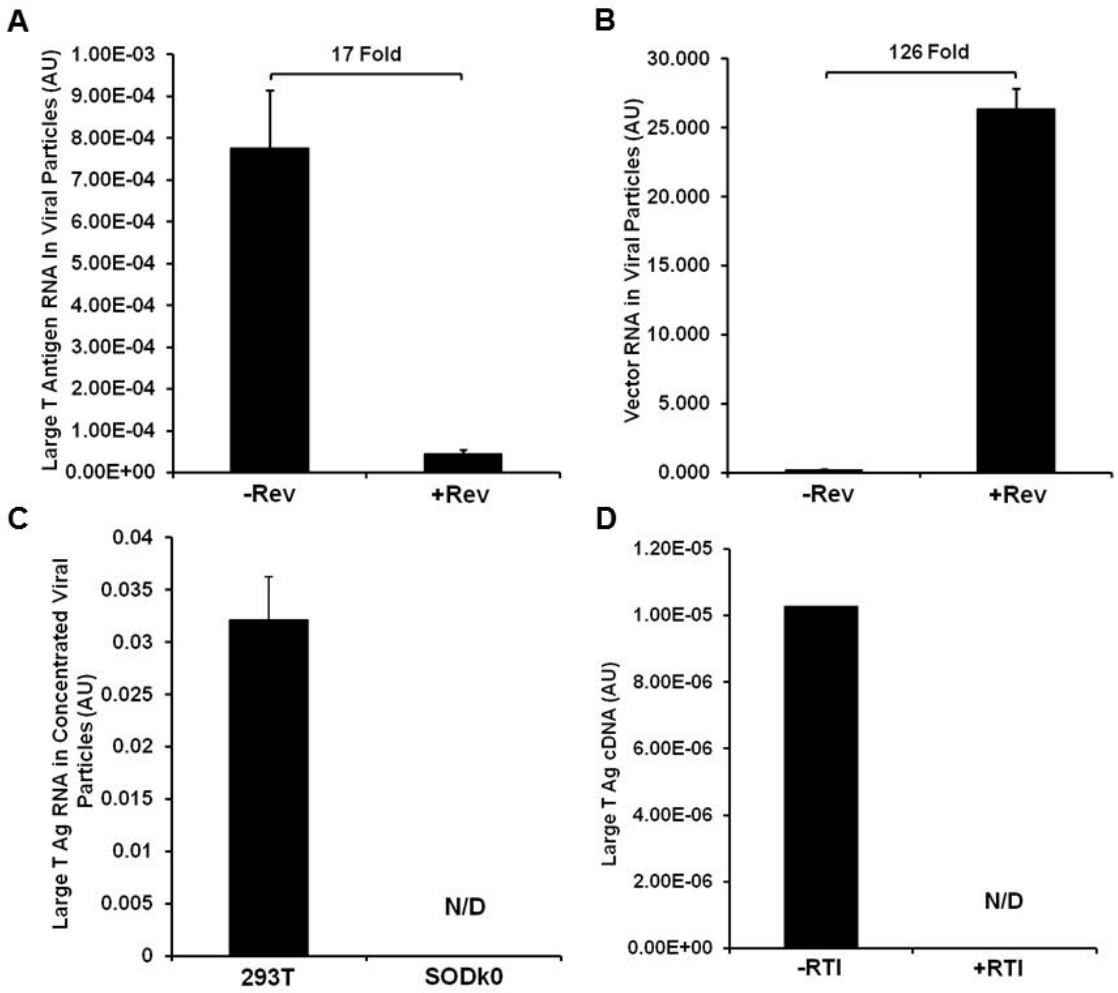


Figure 31. The Rev/RRE system confers specific packaging of HIV-1 vector RNA into HIV-1 viral particles compared to cellular RNA from 293T producer cells. A. RNA packaged into HIV-1 viral particles (vector and cellular) was isolated from equivalent amounts of p24 in the media of 293T producer cells. RNA levels were measured by qRT-PCR. RNA levels for Large T antigen are shown in the absence and presence of Rev. **B.** Examining the same viral particles as those in part A vector RNA levels were measured by qRT-PCR in the absence and presence of Rev. **C.** RNA packaged into HIV-1 viral particles (vector and cellular) was isolated from equivalent amounts of p24 as determined from concentrated vector particles. Vector particles were produced and concentrated from 293T cells, or SODk0 cells. Large T antigen RNA was measured by qRT-PCR (detection was at 29-30 C_t). **D.** HIV-1 vector was packaged into HIV-1 viral particles in the presence of Rev. 293T cells were transduced with equivalent amounts of p24 in the absence or presence of the HIV-1 specific reverse transcriptase inhibitor, etravirine (100nM). Total DNA was harvested at 24 hours post-transduction. Relative large T antigen cDNA levels were measured by qPCR, and normalized to β-globin levels (detection was at 37-38 C_t). All data are expressed as arbitrary units (AU). N/D respresents no detectable level of RNA as determined by Ct values out to 40 cycles.

Chapter 4

UNIQUE TRANSDUCTION PROPERTIES OF HETEROLOGOUS CHIMERIC VECTOR RNAs DELIVERED BY HIV-1 VIRAL PARTICLES[1]

Introduction

Work presented in chapters 2 and 3 demonstrate that the Rev/RRE system can enhance encapsidation of RNA derived from MLV, FIV, and EIAV retroviruses, into HIV-1 derived viral particles. The combination of the Rev/RRE system and *cis* elements from the 5' UTR synergistically augmented heterologous MLV vector titers yielding transduction efficiencies that approach those of standard HIV-1 vectors. Additionally, the heterologous MLV vector was subject to inhibition by a HIV-1 specific reverse transcriptase inhibitor, demonstrating that transduction was dependent on reverse transcription by the HIV-1 reverse transcriptase. In fact, all heterologous-derived vector RNAs (MLV, EIAV, & FIV) were competent for transduction of host cells mediated by HIV-1 structural and enzymatic proteins, indicating that these systems may also serve as a model system to investigate the functional contributions of *cis* elements and *trans* factors that influence host cell transduction. Moreover, data in chapter 3 indicate that the chimeric MLV/HIV vectors do not integrate following delivery with HIV-1 derived viral particles (Fig. 22). These results signify that the chimeric vectors can deliver a transgene as an episomal (nonintegrating) lentiviral vector, with potential for use in gene therapy protocols.

[1] A portion of the work in this chapter was published by Adam Cockrell, Henriette van Praag, Nicholas Santistevan, Hong Ma, and Tal Kafri, titled:The HIV-1 Rev/RRE system is required for HIV-1 5' UTR *cis* elements to augment encapsidation of heterologous RNA into HIV-1 viral particles (45).

In the last decade the use of HIV-1 vector episomes for therapeutic gene delivery has garnered much attention as a means to improve retroviral vector safety by reducing the potential for genotoxicity arising from an unintended event of vector integration (11, 210). Early clinical trials, employing integration competent simple retroviral vectors delivered by MLV viral particles, revealed the potential to successfully correct children with the genetic SCID-X1 disease; however, the development of T cell leukemia in 3 of 10 patients, revealed genotoxicity effects associated with simple retroviral vector integration (75, 76, 157). Integration competent HIV-1 derived vectors were pursued as a safer alternative for clinical trials. Two recent clinical studies showed benefit to patients receiving therapeutic transgenes delivered by HIV-1 vectors (29, 30). However, clinical follow-up in one of the studies revealed a myeloid clonal dominance (30), revealing the potential for HIV-1 vector-mediated genotoxic effects. Averting genotoxic effects of vector integration may be realized by two different strategies: i) targeting integration to specific sites within the host genome; and ii) exploiting non-integrating episomal retroviral vector cDNAs for gene delivery (11, 210). Targeted integration would be important for therapeutic gene delivery to dividing cells *ex vivo*, such as in the clinical studies mentioned above. In the event that transient gene expression is required for treatment in dividing cells, episomal vectors may be applicable. However, numerous genetic diseases, such as those of the eye, central nervous system (CNS), muscle, and liver may require *in vivo* administration to tissues predominantly consisting of non-dividing cells. HIV-1 derived episomes are especially stable in non-dividing cells, and are only diluted upon cell division (23, 25, 175), making them desirable for gene therapy.

Episomal DNA forms comprise the majority of detectable cDNAs following transduction, and they include: 2-LTR circles that form from non-homologous end joining of a linear proviral cDNA; 1-LTR circles that can be generated directly as a consequence of the reverse transcription process, or through homologous recombination of the LTR's; linear proviral cDNAs that are not competent for integration; and mutant proviral DNAs that may be generated by self-integration (34, 46, 169, 203). HIV-1 derived episomal vector formation is typically promoted through use of integrase-defective mutants (14, 99, 137, 173, 179, 207, 219), and vectors with mutations in the attachment (*att*) sites at the boundaries of the vector LTRs (6, 158). Despite the improved safety profile of episomes to diminish the potential for insertional mutagenesis, linear cDNA episomal products remain the dominant species, and have the potential to instigate genotoxicity through illegitimate integration (i.e. proviral DNA integration that is not mediated by a functional integrase). Since the linear episomal species are the products of a successful reverse transcription process, it was recently realized that interfering with reverse transcription can alter the profile of episomal vector species to favor 1-LTR circles (99), thereby ameliorating the episome safety profile. The ability to manipulate the episomal profile to direct formation of a particular cDNA species would be favorable. In addition to therapeutic gene delivery, retroviral vectors provide a platform to investigate the molecular virology occurring at different steps of transduction.

HIV-1 delivered chimeric MLV/HIV vectors exhibit a transduction profile consistent with episomal vector cDNA species (chapter 3), but without modifying the HIV-1 integrase or existing MLV *att* sites. Heterologously packaged vectors may not only be desired for therapeutic gene delivery, but can also be of benefit as a model system to understand the molecular mechanisms that underlie early events of retroviral infection. In the studies

presented here chimeric MLV/HIV, FIV/HIV, and EIAV/HIV vectors were heterologously packaged into HIV-1 viral particles to investigate HIV-1 components that confer efficient and stable transduction. The studies demonstrate that: i) HIV-1 *trans* factors (structural and enzymatic proteins) are sufficient to deliver heterologous MLV, FIV, and EIAV cDNA species to non-dividing, and dividing, cells *in vitro* and *in vivo*; ii) HIV-1 integrase is sufficient to recognize FIV *att* sites to support stable transduction of heterologous FIV cDNAs, but not EIAV or MLV; iii) stable transduction of cDNAs derived from chimeric EIAV/HIV vector RNAs lack the proper *att* sites for HIV-1 mediated integration; iv) episomes derived from heterologously packaged MLV/HIV chimeric RNAs are predominantly generated by reverse transcription into 1-LTR cDNA species without manipulating *att* sites or HIV-1 integrase; and v) HIV-1 mediated transduction of heterologous MLV/HIV chimeric cDNA species requires not only the proper *att* sites, but more importantly appropriate *cis* elements (PBS & PPT) for reverse transcription to generate a linear cDNA species competent for integration; vi) HIV-1 appears to share a significant degree of functional conservation with the non-primate FIV lentivirus, but not EIAV or MLV; and, vii) EIAV/HIV and MLV/HIV vectors exhibit *in vivo* transduction properties that may be exploited for therapeutic gene delivery.

Methods

Plasmid Constructs. Murine leukemia virus (MLV) and MLV/HIV RRE + RU5PS chimeric vector constructs were generated as described in chapters 2 & 3 methods. The MLV/HIV RRE vector (pTK1086) described in Fig. 33 was an earlier generation of pTK1332 above, which did not have the luciferase gene inserted. The pTK terminology for plasmid constructs

refers to the plasmid library in the Kafri laboratory. Chimeric MLV/HIV vectors with modified attachment (*att*) sites, PBS, and PPT, are a derivative of pTK1086 described as follows. The following cloning series was used to generate the MLV/HIV *att* vector: i) a *MluI/BstEII* fragment, containing the MLV 5' LTR from the MLV vector pTK506 was subcloned into pSL301 (Invitrogen) to generate pTK682; ii) pTK682 was used as a template for PCR around the entire plasmid wherein primers were designed to change the MLV *att* site to a HIV-1 *att* site, the region between the *MluI/BstEII* sites was confirmed by sequencing to be correct, an MLV 5' LTR with HIV-1 *att* sites, pTK1349; iii) the *MluI/BstEII* fragment from pTK1349 was cloned into the corresponding region of pTK1086 to generate pTK1352 (a MLV vector with a 5' LTR containing the HIV-1 *att* site and 3' LTR containing the MLV *att* site); iv) to alter the 3' LTR a *SapI/SacII* fragment from pTK1086 was inserted into pBlueScript, pTK1348, and this was used as a template for PCR around the entire plasmid wherein primers were designed to change the MLV *att* site to a HIV-1 *att* site, pTK1350; and, v) the *SacII/SapI* fragment from pTK1350 was cloned into the corresponding sites in pTK1352 to generate the MLV/HIV *att* construct termed pTK1354. The following cloning series was used to generate the MLV/HIV *att* + PPT vector: i) pTK1348 was used as a template for PCR around the entire plasmid wherein primers were designed to change the MLV *att* and PPT sites to those of HIV-1 (pTK1351) which was sequence verified between *SacII* and U5 in the vector; and ii) the *SacII/SapI* fragment from pTK1351 was subcloned into pTK1352 to generate the MLV/HIV *att* + PPT chimeric vector, pTK1355. The MLV/HIV att + PBS construct was prepared with the following cloning sequence: i) a SacII/SapI fragment from pTK1350 was cloned into pTK1086 to generate a chimeric vector with a single HIV-1 att site in the 3' LTR, pTK1356; ii) pTK682 was used as a template for

PCR around the entire plasmid wherein primers were designed to change the MLV *att* site and PBS to those of HIV-1 *att* site and PBS, and the region between the *MluI/BstEII* sites was confirmed by sequencing to be correct, an MLV 5' LTR with HIV-1 *att* & PBS sites, pTK1353; iii) The *MluI/BstEII* fragment from pTK1353 was inserted into the corresponding sites of pTK1356 to generate the final chimeric vector MLV/HIV *att* + PBS, pTK1358. The final MLV/HIV chimeric vector, MLV/HIV *att* + PBS + PPT, was generated with the following subclonings: i) a *SacII/SapI* fragment from pTK1351 was inserted into the corresponding sites of pTK1086 to generate pTK1357; and ii) a *MluI/BstEII* fragment from pTK1353 was inserted into the corresponding sites in pTK1357 to yield the final chimeric vector MLV/HIV *att* + PBS + PPT, or pTK1359.

Construction of the parental EIAV and FIV, and the chimeric EIAV/HIV RRE and FIV/HIV RRE, vectors is described in the chapter 2 methods. The EIAV/HIV *att* + RRE vector was constructed as follows: i) for modification of the 3' LTR *att* a *NaeI* fragment from pTK728 was cloned into a standard cloning vector in the lab, pTK50, to generate pTK1242; ii) pTK1242 was used as a template for PCR amplication around the entire plasmid thereby replacing the EIAV *att* site with the HIV-1 *att* site, and sequence was confirmed to generate pTK1345; iii) similarly for modification of the 5' LTR *att* a *BglII/NotI* fragment from pTK728 was cloned into the corresponding sites of pTK50 to generate pTK1241; iv) pTK1241 was used as a template for PCR amplification around the entire plasmid thereby replacing the EIAV *att* site with the HIV-1 *att* site, and sequence was confirmed to generate pTK1344; v) the *BglII/NotI* fragment from pTK1344 was inserted into pTK728 to generate pTK1346; and vi) the final construct was generated by inserting the *RsrII/PacI* fragment from pTK1345 into pTK1346, yielding the EIAV/HIV *att* + RRE vector, pTK1347. The

chimeric EIAV/HIV RRE vector containing the liver specific promoter (hAAT) driving expression of the firefly luciferase gene was constructed as follows: i) The parental EIAV vector UNC 6.1 W-1 was cut with *NheI/XbaI* and self-ligated to generate pTK827; ii) a *NotI/SacII* fragment from pTK208 was inserted into the respective sites in pTK827, thereby constructing pTK829; and iii) a *XhoI/NotI* fragment from pTK647 containing the hAAT-FLuc expression cassette, was inserted into the corresponding sites of pTK829, yielding the final construct pTK857.

The packaging constructs supplying necessary structural/enzymatic proteins were 4XCTE or ΔNRF as described in chapter 2 methods. The Rev and VSV-G expression constructs are explained in chapter 2 methods.

Cells, Viral Particle Production, and Concentration. All are described in chapter 2 methods.

HIV-1 p24 Capsid Concentration. Details of this assay were described in chapter 2 methods.

MLV Reverse Transcriptase Assay. Details of this assay were described in chapter 3 methods.

FACS Analysis. Details of this assay were described in chapter 2 methods.

Southern Blot Analysis. 293T cells were transduced with indicated chimeric vectors packaged into HIV-1 or MLV viral particles, at MOI's of 5 or 10. Total cellular DNA was isolated at 5 days post-transduction (referred to as P0, or no cell passages, in figures), or after five passages (P5) of the transduced cells where indicated. To collect total DNA transduced cells were lysed in a proteinase K solution (10 mM Tris pH 8, 10 mM EDTA pH 8, 0.5% SDS, 0.4 M NaCl, and 200 µg/ml proteinase K) for 48 hours at 55°C. Total DNA was

extracted with v/v phenol (Invitrogen) and v/v phenol/chloroform/isoamyl alcohol (Invitrogen) and then treated with RNase A (20 μg; Fermentas) at 37°C for 2 hours. A second round of extractions was performed and total DNA was precipitated using standard ethanol precipitation.

Regarding studies with the MLV/HIV RRE + RU5PS chimeric vector isolated gDNA (10 μg) was digested with BsrGI and DpnI for ~24 hours at 37°C. The gDNA (15 μg) from transductions with MLV chimeric vectors with modified *att*, PBS, or PPT sites were digested with PflMI, SexAI, and DpnI for ~24 hours at 37°C. The gDNA (15 μg) from transductions with chimeric EIAV vectors were digested with PvuII, PflMI, and DpnI for ~24 hours at 37°C. DpnI was included to eliminate putative plasmid DNA carry-over from the transfections during vector production in 293T cells. Equivalent amounts of gDNA were resolved on 1% agarose gels, transferred to Zetaprobe membrane, and probed to the 5' or 3' ends of the vector, where indicated, enabling a size distinction between linear, 1-LTR, 2-LTR and backbone/integrated forms. Images were captured on BioMax MR Film (Kodak), or by phosphorimager (Molecular Dynamics Storm System).

Real Time qPCR. Total cellular DNA was isolated as described for Southern blot analysis and qPCR for vector and β-globin were described previously (100). Briefly, vector copy number was derived from standard curve produced from FLP9 cells, which contain a single copy of HIV-1 vector per diploid genome. One nanogram of total cellular DNA was previously calculated to contain 303 copies for β-globin per diploid genome, or 151.5 copies vector per diploid genome. Primers for vector copy number were designed to the WPRE region, which is also in our MLV/HIV chimeric vectors. All primers and PCR reaction conditions were described previously (100).

Transduction of aphidicolin-arrested 293T cells. *In vitro* experiments to arrest 293T cells in the cell cycle were treated 24 hours prior to transduction with 5μg/ml aphidicolin in 6 well plates, thereby arresting at the G1/S phase of the cell cycle. Arrested cells were transduced 24 hours after aphidicolin arrest with 2μl ($4x10^5$ TU/μl) of the indicated vectors to examine transduction of non-dividing cells. 293T cells in the absence of aphidicolin were transduced with the same volume at the same time that aphidicolin was added to the plates to allow more time for detection of GFP expression. Transduced cells were imaged by fluorescence microscopy at 48 hours post-transduction for aphidicolin treated cells.

***In vivo* experiments in the mouse liver.** All care and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. [NIH]85-23), and all procedures received prior approval by the University of North Carolina Institutional Animal Care and Usage Committee. Balb/c mice were injected intraperitoneally (IP) at 8-10 weeks of age with equivalent p24 amounts (~100 μg) of a single concentrated preparation of EIAV/HIV RRE chimeric vector packaged into HIV-1 viral particles (i.e. a single large concentrated preparation was equally divided over two mice). Mice were examined for luciferase expression in the liver at 3 and 23 weeks post-injection. Luciferin (Promega), 125mg/kg, was administered IP and measured using the Xenogen IVIS imaging system (Xenogen, Hopkinton, MA).

Generating transgenic animals. All care and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. [NIH]85-23), and all procedures received prior approval by the University of North Carolina Institutional Animal Care and Usage Committee. The generation of all transgenic animals was executed under the direction of Randy Thresher and Kimberly Kluckman within the Animal Transgenic Core

facility at the University of North Carolina. Single cell embryos were microinjected into the perivitelline space with each of the indicated vectors at 1-100 IU/embryo. Embryos were implanted into a pseudopregnant female by 2 days after microinjection of vector. Embryos were imaged prior to implanting by fluorescence microscopy. Mice were imaged for GFP expression at 16-20 weeks of age. A defined region of the mouse abdomens were cleared of hair. Mice were anesthetized with avertin (0.4-0.7mg/g) prior to imaging with a digital camera using a GFP filter. Of the animals imaged 10% of the heterologous FIV/HIV transgenic mice were positive for GFP expression. Genomic DNA was extracted from tail clipping of adult mice and assessed for presence of proviral vector DNA. Standard PCR was performed on the genomic DNA with primers designed to the WPRE for vector and endogenous mouse β-actin.

Rat Subjects and Stereotaxic Administration of Retroviral Vectors. Pathogen-free Sprague-Dawley rats were obtained from Charles Rivers. All care and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. [NIH]85-23), and all procedures received prior approval by the University of North Carolina Institutional Animal Care and Usage Committee. Virus vector infusions and animal perfusions were performed in the laboratory of Dr. Thomas McCown at the University of North Carolina, as previously described (141). Briefly, rats were anesthetized with 50mg/kg pentobarbital and then placed into a stereotaxic frame. Using a 32 gauge stainless steel injector and Sage infusion pump, the rats received 3µl (at $4x10^5$ GFP TU/µl) of MLV/HIV RRE chimeric vector (pTK1086) packaged into MLV or HIV-1 viral particles, over a 30 minute period into the striatum (1.0mm anterior to bregma, 3.0mm lateral, 5.5mm vertical) according to the atlas of Paxinos and Watson (171). 6 rats were bilaterally infused with each

vector at the same time (i.e. HIV-1 packaged vector was injected into the right striatum, and concurrently MLV packaged vector was injected into the left striatum). In the instance of chimeric FIV/HIV RRE vector (pTK660) and HIV-1 vector (pTK113) 1μl was injected over a 10 minute period into the striatum.

At 10 days (chimeric FIV vector experiements) or 1 month (chimeric MLV vector experiments) after vector infusion, rats received an overdose of pentobarbital (100 mg/kg i.p.) and subsequently perfused transcardially with ice-cold 100mM PBS (pH 7.4), followed by 4% paraformaldehyde in 100mM phosphate buffer (pH 7.4). After overnight fixation in paraformaldehyde-phosphate buffer, 40 μm vibratome sections were prepared through the striatum and rinsed in phosphate buffer.

Mouse Subjects and Stereotaxic Administration of Retroviral Vectors. Mice viral vector injections and tissue preparation were executed in the laboratory of Dr. Henriette van Praag at the National Institute on Aging. Female C57Bl/6 mice (n=10), 3 months old (Jackson Labs) were housed in standard conditions with 3-4 mice per cage with food and water available *ad libitum*. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (National Institutes of Health) 85-23), and all procedures received prior approval by the NIH Institutional Animal Care and Usage Committee. All procedures were performed according to animal protocol number, 396-LNS-2014.

Mice were anesthetized (Avertin 0.02 mg/ml, 0.5-1.0 ml injection per mouse), and MLV retrovirus (n=5 mice), or HIV lentivirus (n=5 mice) was injected stereotactically (2 μl [$4x10^5$ TU/μL] using a 5 μl Hamilton microliter syringe) into the right striatum (AP= 1.0 mm anterior from bregma; lateral= 1.5 mm; ventral=3.0 mm). Studies were executed with the

MLV/HIV RRE vector packaged into MLV viral particles, or HIV-1 viral particles generated from the ΔNRF helper construct. One month thereafter animals were given an overdose of anesthetics and perfused transcardially with cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). After 24 hr, brain tissue was equilibrated in 30% sucrose. Sequential horizontal sections (40 μm) using a sliding freezing microtome (HM450, ThermoFisher) were taken through the extent of the striatum and stored in phosphate buffered glycerol at -20˚C.

Immunohistochemistry and Confocal Microscopy. Immunohistochemistry for mouse studies was executed in the laboratory of Dr. Henriette van Praag at National Institute on Aging. For mouse studies double labeling for the neuronal marker NeuN and GFP was done on a 1:6 series of 40-μm free-floating horizontal sections as described previously. Sections were washed and blocked in TBS with 3% donkey serum and 0.3% Triton X-100 (TBS-plus). Primary antibodies raised in two different species were pooled in TBS-plus and incubated for 48 h at 4°C. The neuronal marker NeuN [mouse monoclonal antibody, (Millipore) 1:100] was combined with antibody for GFP [rabbit polyclonal antibody (Millipore), 1:1000]. Corresponding secondary antibodies [donkey anti-mouse CY3 and goat anti-rabbit Alexa488 (Jackson ImmunoResearch Inc.), 1:250] were pooled, and sections were incubated for 4 h at room temperature following washing in TBS-plus. Sections were mounted and coverslipped with DABCO-PVA.

For rat studies immunohistochemisty was executed under the guidance of Dr. Bonnita Blake in the laboratory of Dr. Thomas McCown at the University of North Carolina. For immunohistochemistry, tissue sections were incubated in 10% normal goat serum and 0.1% Triton X-100 in PBS for 45 minutes. Sections were then incubated with primary antibody to

NeuN (1:500, Chemicon) or rabbit anti-GFP (1:500, Millipore) overnight in 3% normal goat serum, 0.2% Triton X-100 and PBS. Tissue sections were rinsed in PBS, incubated in blocking serum (10% normal goat serum, 0.1% Triton X-100, PBS) for 1 hour and then incubated with a secondary antibody (Alexa-fluor 594 goat anti-mouse for NeuN [red, Molecular Probes] and goat anti-rabbit Alexa-fluor 488 to detect GFP [Green, Invitrogen]) for 1 hour at 4°C. Following 3 rinses in PBS, the sections were mounted on slides and coverslipped with fluorescent mounting media. For highly sensitive detection of GFP expression by DAB staining in the rat striatum tissue sections the primary antibody was a rabbit anti-GFP-biotinylated antibody (1:500, Chemicon). The secondary staining was performed according to instructions using a secondary antibody kit (Vectastain Elite ABC kit, Vector Laboratories). The DAB staining solution (resuspend lyophilized powder in 10 ml water and add to the following: 10 ml 0.2M PBS + 175 µl $NiNH_2SO_4$ + 200 µl $CoCl_2$ + 5 µl 3% H_2O_2 and filter through 0.2 µm filter) was added to the tissues for 10-15 minutes at room temperature, carefully monitoring the color of the samples not to turn overly dark. The samples were then washed three times with PBS and mounted onto slides using a toluene based liquid, TBS SHUR/mount.

DAB stained striatum rat brain sections were imaged using a slide analyzer and Aperio ImageScope version 10.0 software. For colocalization studies by fluorescence detection sections were imaged by confocal scanning laser microscopy (MPE1000, Olympus and Laser Scanning Zeiss 510 Meta). Mouse and rat brain sections with GFP positive cells were captured with the Zeiss LSM Image Browser software and used to score for GFP, NeuN, and colocalization of the two markers by scanning through the z-axis. Scoring represents positive cells from brain sections of mice, or rats (n=6, for bilateral injections),

transduced with the MLV/HIV RRE vector packaged into MLV (n=5, for mice), or HIV-1 (n=5, for mice), derived viral particles.

Results

HIV-1 *trans* factors facilitate *in vitro* and *in vivo* transduction of heterologously packaged MLV RNA through generation of episomal cDNA species. Depending on the type of viral particle carrying a heterologous vector RNA (HIV-1 or parental viral particles), a different transduction profile may be anticipated. The transduction profile is indicative of molecular mechanisms that govern the various stages of the transduction process (attachment, entry, uncoating, reverse transcription, nuclear entry, integration, and gene expression) in a host cell. In the context of HIV-1 viral particles GFP positive cells from transduction with HIV-1 delivered chimeric MLV/HIV vector were almost completely eliminated (Chapter 3, Fig. 22), indicating that GFP may be expressed from episomal DNA vector forms not competent for integration. In contrast, in the context of MLV viral particles the chimeric vector was retained after multiple cell passages to eliminate episomal vector DNA species, as indicated by FACs analysis of GFP positive cells (only about 50% reduction, Fig. 32A). The presence of total episomal DNA forms was assessed by qPCR for vector copy number (Fig. 32B) relative to copy number of β-globin (Fig. 32C). In line with GFP results, qPCR quantitation of total vector DNA before and after passaging cells yielded a >30 fold decrease in vector DNA after passaging 293T cells transduced with HIV-1 particles, compared to <2 fold decrease from MLV viral particles (Fig. 32D). MLV/HIV chimeric vector RNA delivered by HIV-1 viral particles primarily cedes proviral episomal DNA forms that can be composed of 1-LTR, 2-LTR, linear, and mutant forms.

Nonintegrating cDNAs are readily lost as a consequence of dilution during cell division. However, anticipated therapeutic applications would comprise transgene delivery to the preponderance of non-dividing/slowly dividing cells, as previously demonstrated with non-integrating HIV-1 derived vectors delivered to the eye, CNS, muscle, and liver (6, 14, 99, 137, 173, 179, 219). Unique to lentiviral vectors is the ability to establish stable transgene expression in non-dividing cells, a barrier encountered by MLV derived vectors delivered by MLV viral particles. *In vitro* delivery of a MLV/HIV RRE chimeric vector (Fig. 33A) by HIV-1 viral particles exhibited efficient transduction on 293T cells arrested with aphidicolin in the G1/S phase of the cell cycle (Fig. 33B). In contrast, transduction was almost completely absent following delivery of the same vector with MLV viral particles (Fig. 33B). *In vitro* transduction of non-dividing cells implies that HIV-1 structural and enzymatic proteins are sufficient to ferry an MLV derived vector cDNA species into the nucleus for subsequent transgene expression. Nonetheless, the 'Gold Standard' for HIV-1 derived vector is *in vivo* transduction of quiescent neurons in striatum of mouse and rat brains.

HIV-1 mediated delivery of a MLV/HIV RRE chimeric vector that expresses GFP (Fig. 33A) demonstrated efficient transduction of mouse brain neurons in the striatum (Fig. 34A and B). HIV-1 delivered vector (GFP marker) colocalized in neurons stained for the neuronal nucleus (NeuN) marker, whereas the same vector delivered with MLV viral particles did not exhibit neuronal transduction (Fig. 34A and B); an observation that was consistent over multiple brain sections (Fig. 34B). Nonetheless, MLV delivered vector did yield a moderate number of GFP positive cells that were not NeuN positive and appeared to be morphologically distinct. Similar results were obtained following delivery to the rat brain striatum. The extent of transduction in three separate rat brains was observed by indirectly

staining for GFP transgene expression from the MLV/HIV chimeric vector using a secondary antibody amenable to DAB staining (Fig. 35). This staining method is also highly sensitive allowing for detection of low expressing GFP positive cells. The morphology of the dark stained cells is consistent with those of neurons, demonstrating HIV-1 viral particles can effectively deliver the MLV chimeric vector to neurons. Further affirmation came from confocal microscopy studies showing colocalization of cells for GFP expressed from the chimeric MLV/HIV vector (green) and the NeuN marker (red), only when the vector was delivered by HIV-1 derived viral particles (Fig. 36A & B). Quantification from six rat brains demonstrated nearly 100% transduction of neurons when the chimeric MLV/HIV vector was delivered by HIV-1 viral particles (Fig. 36B). However, in contrast to the data observed for the mouse transduction there were as many GFP positive cells (i.e. vector positive) upon delivery with the MLV viral particles (Fig. 36B). These cells appeared to be morphologically small, and localized primarily at the injection site (Fig. 36A). These may be a type of lymphocyte that can readily divide, thereby amenable to transduction by MLV viral particles, but would not be detected in brains were the chimeric vector is delivered by HIV-1 viral particles since the episomes would be diluted upon cell division. Nonetheless confirmation would require further characterization. Additionally, a higher level of neurons transduced by MLV delivered chimeric vector was also apparent in the rat brain striatum (Fig. 36B). The distinction between MLV delivery in the rat and mouse brains may be explained by differences in delivery. In the mouse studies vector was administered to the striatum of only one brain hemisphere, whereas in the rat study MLV and HIV-1 packaged vectors were bilaterally administered into opposing brain hemispheres of the same animal. Feasibly, concomitant insult to both hemispheres may provoke cellular migration or leakage across the

brain hemispheres. Further discourse on this topic can be obtained below. Overall, these data are a clear indication that heterologous MLV vector RNAs, packaged into HIV-1 viral particles, can assume transduction properties dictated by HIV-1 structural and enzymatic proteins; except, however, that reverse transcribed cDNAs cannot integrate, therefore transgene expression is primarily from episomes.

HIV-1 *trans* factors facilitate *in vitro* and *in vivo* transduction of heterologously packaged EIAV and FIV RNAs. Chimeric EIAV/HIV and FIV/HIV vectors, packaged into HIV-1 viral particles, were demonstrated to exhibit efficient *in vitro* transduction of 293T cells as determined by vector titers (chapter 2). Delivery of each chimeric vector was also examined through *in vivo* administration to mice. A chimeric EIAV/HIV vector was generated with an expression cassette composed of the liver specific human alpha antitrypsin (hAAT) promoter driving expression of the firefly luciferase (FLuc) gene (Fig. 37A). HIV-1 viral particle (ΔNRF) delivery of the chimeric EIAV/HIV vector by intraperitoneal injection exhibited strong luciferase expression at 3 weeks post-injection. However, expression was significantly reduced by 23 weeks post-injection, as indicated by the necessity to increase the detection sensitivity 5 fold (Fig. 37B). These data are consistent with the possibility that the EIAV/HIV chimeric vector is similar to the MLV/HIV chimeric vectors in that expression is primarily from episomal cDNA forms. Moderate cell division that occurs in the liver may gradually result in a reduction in detectable signal as non-integrated cDNAs are lost to dilution effects.

HIV-1 mediated delivery of chimeric FIV/HIV vectors exhibit *in vivo* transduction properties that are more similar to HIV-1 derived vectors, rather than MLV/HIV and

EIAV/HIV chimeric vectors. A chimeric FIV/HIV vector containing a CMV-GFP expression cassette (Fig. 38A) was delivered by HIV-1 viral particles to mouse brain striatum (Fig. 38B), and to mouse embryos ultimately resulting in the generation of GFP expressing transgenic mice (Fig. 38C & D). Similar to the MLV/HIV chimeric vector, the FIV/HIV chimeric vector was readily transferred to neurons in mouse brain striatum (Fig. 38B), as determined by morphological analysis. More importantly, however, is the fact that transgenic mice could be generated through HIV-1 mediated delivery of the chimeric FIV/HIV vector (Fig. 38C & D). Since numerous cell divisions are required to generate a transgenic mouse from an embryo these data were a clear indication that HIV-1 integrase could mediate integration of the chimeric FIV/HIV vector, which is in contrast to what was observed for chimeric MLV/HIV and EIAV/HIV vectors. Confirmation of these distinct transduction profiles was obtained by transducing 293T cells with each of the chimeric vectors (in this case the EIAV/HIV chimeric vector contains a CMV-GFP expression cassette), and measuring the percent of cells that were positive for GFP expression before and after passaging the cells (Fig. 39). The chimeric MLV/HIV RRE + RU5PS and EIAV/HIV RRE vectors were diluted upon passaging the cells, an indication that both are primarily in the form of episomes in transduced cells. The FIV/HIV RRE chimeric vector, on the other hand, exhibited a high frequency of integration insinuating at least partial recognition and function by the HIV-1 reverse transcriptase and integrase proteins.

HIV-1 reverse transcriptase and integrase utilize distinct molecular mechanisms to promote chimeric EIAV and MLV episome formation. It is well accepted that stable transduction of dividing cells is dependent upon successful integration of a viral/vector linear

DNA. The main viral components that facilitate this process are integrase and the appropriate attachment (*att*) sites at the ends of the linear cDNA, which are recognized by the viral integrase (Fig. 40). However, the generation of a linear cDNA competent for integration requires proper reverse transcription. As previously discussed the cDNA products of reverse transcription are not only linear molecules of cDNA capable of integration, but the majority are comprised of different types of episomal DNA forms including 2-LTR, 1-LTR, and linear molecules that do not integrate (Fig. 40). Mutant circular molecules may also arise as a consequence of auto-integration. Aforementioned data suggests that the chimeric EIAV/HIV and MLV/HIV vector cDNAs are in the form of episomes, however the types of episomes present was not clear from these studies. Southern blot analysis revealed that the chimeric EIAV/HIV RRE vector is predominantly comprised of linear episomes, indicating that the HIV-1 reverse transcriptase generates linear cDNA vector molecules competent for integration (Fig. 41C). However, since stable transduction is not easily attainable, the HIV-1 integrase may not properly recognize the EIAV *att* sites. EIAV *att* sites were replaced with those of HIV-1 (Fig. 41A), demonstrating a 9 fold increase in stable transduction following dilution of episomal DNA forms by passaging the cells (Fig. 41B). Moreover, changing the *att* sites only moderately altered the episomal profile, possibly reducing the amount of 2-LTR circles formed by delivery with HIV-1 viral particles (Fig. 41C). The profiles of both vectors when packaged into EIAV viral particles was similar, with the exception that the total amount of DNA is less in the lane representing the vector without *att* sites (Fig. 41C). These data demonstrate that the HIV-1 *att* sites were sufficient to enhance stable transduction nearly 10 fold, by providing the appropriate substrate for the HIV-1 integrase.

The episomal profile of the EIAV/HIV chimeric vector indicated that replacing the *att* sites would be sufficient to confer stable transduction through proper recognition by the HIV-1 integrase. The molecular mechanisms, however, extend beyond *att* site recognition by integrase to include proper reverse transcription. A previous study indicated that deletion of the 3' PPT from an HIV-1 derived vector shifted the episome profile from linear cDNA products to a dominant 1-LTR circle form (99). Interestingly, Southern blot analysis revealed that the HIV-1 packaged chimeric MLV/HIV RRE+RU5PS vector (Fig. 42A) exists predominantly as a 1-LTR episome that can be diluted following multiple cell divisions through passaging (Fig. 42B). A very similar profile was observed using a 3' probe, with a minor amount of detectable linear forms (Fig. 42C), indicating that minuscule amounts of linear species of various sizes may be present but not competent for integration. In contrast, the same vector delivered with MLV viral particles exhibited a very different profile, primarily as integrated DNA accompanied by minimally detectable levels of linear, 1-LTR, and 2-LTR episomes (Fig. 42B). These data are in accordance with what was observed in figure 32D. The absence of competent linear episomal forms indicates that reverse transcription of the chimeric MLV/HIV vector RNA by HIV-1 RT clearly leads to dominant 1-LTR episomal species that are apparently responsible for the observed GFP expression. Furthermore, it can be anticipated that replacing the *att* sites would not be sufficient to recover integration, but rather replacing the MLV PPT & PBS with those from HIV-1 would facilitate proper reverse transcription of the chimeric MLV/HIV vector RNA into appropriate linear forms that may then be competent for stable transduction in the presence of the HIV-1 *att* sites. Indeed, replacing the MLV *att* sites with those of HIV (Fig. 43A) yielded a minor increase in stable transduction as measured by FACS analysis for GFP positive cells (Fig.

43B). Additional modifications to an MLV/HIV RRE chimeric vector included replacing the MLV PBS & 3' PPT with those of HIV-1 in the context of HIV-1 *att* sites (Fig. 43A). Although independently the PBS or 3' PPT exhibited a significant increase in stable transduction, the most dramatic influence was the combined effect of both to yield a >20 fold increase in stable transduction (Fig. 43B). The episomal cDNA profiles of these vectors were examined by Southern blot analysis (Fig. 44). Blots were probed with either a 3' probe (Fig. 44B) or 5' probe (Fig. 44C). The 3' probe not only revealed the presence of 1-LTR episomal forms but also that there are indeed linear forms present, but apparently not the correct linear forms (i.e. linear molecules competent for integration by the HIV-1 integrase) since linear forms are nearly absent after probing with the 5' probe. Apparently, the process of reverse transcription generates randomly sized linear products at the 5' end (Fig. 44A) that are truncated to a defined size after cutting with the indicated enzymes, and can only be detected with the 3' probe. Importantly, however, in the presence of the HIV-1 3' PPT there is an increase in linear products detectable by the 5' probe (Fig. 44C); an indication that the HIV-1 reverse transcriptase is able to generate linear cDNA products that are amenable to recognition by the HIV-1 integrase. Overall these data demonstrate that the HIV-1 reverse transcriptase and integrase enzymes can generate distinct episomal profiles for EIAV/HIV and MLV/HIV chimeric vectors.

Discussion

The goal of these studies was to investigate the transduction properties of RNAs heterologously packaged into HIV-1 viral particles with the expectation that these studies would provide insight into the molecular mechanisms governing retroviral transduction of a

host cell. Distinct transduction profiles were observed for three different heterologously packaged RNAs: MLV, EIAV, and FIV. For all heterologously packaged vector RNAs the HIV-1 structural and enzymatic proteins were capable of mediating transduction *in vitro* and *in vivo*, and, most importantly, in non-dividing cells (Figs. 33-39). A well established biological phenomenon is that MLV viral particles cannot transduce quiescent cells (217), thereby indicating that, in the studies shown here (Figs. 33-36), HIV-1 *trans* factors are sufficient for a MLV vector RNA to overcome cell cycle-dependent blocks to transduction. Accordingly, another group showed that the HIV-1 capsid protein has a direct role in the transduction of non-dividing cells (218), and not previously suspected HIV-1 karyophilic viral elements (216). However, although these results demonstrate that HIV-1 *trans* factors could surmount blocks to transduction of non-dividing cells, they could not mediate stable transduction of heterologously packaged MLV or EIAV chimeric vector RNAs (Fig. 39). Whereas, stable transduction of heterologously packaged FIV vector RNA was readily achievable (Fig. 38 & 39). These differences could be distinguished by the potential for the HIV-1 reverse transcriptase to generate an appropriate vector DNA substrate (from heterologously packaged chimeric MLV, EIAV, & FIV RNAs) competent for recognition and processing by the HIV-1 integrase. Although this was partially the case for FIV vector DNA, MLV and EIAV vector DNAs remained as distinct episomal species that could be effectively eliminated by dilution upon cell division (Fig. 32, 39-44).

The generation of a linear vector DNA molecule competent for integration requires the reverse transcriptase and its associated RNase H activity to render the appropriate 5' and 3' ends containing attachment (*att*) sites that can subsequently be recognized and processed by the integrase enzyme. Studies presented here demonstrate that HIV-1 reverse transcriptase

can mediate reverse transcription of heterologously packaged chimeric MLV, EIAV, and FIV vector RNAs. However, FIV was effectively integrated, whereas EIAV and MLV remained as episomes. These results are in accordance with previous reports demonstrating efficient heterologous packaging and propagation of RNA derived from primate lentiviruses (184, 198), as well as the non-primate lentivirus FIV (22), into HIV-1 derived viral particles. These studies indicate that not only are the proper FIV linear vector DNAs generated from reverse transcription, but also that HIV-1 integrase can recognize and process the *att* sites to mediate integration. Further support for this proposition comes from a study demonstrating that HIV-1 could support integration of an HIV-1 virus with 5' and 3' *att* sites altered to those of FIV with up to 70% efficiency of wild-type, whereas replacement with MLV *att* sites was less than 0.5% wild-type levels (136). These data, combined with data in chapter 2 demonstrating that FIV Rev is functionally interchangeable with HIV-1 Rev, provides strong evidence for a significant level of functional conservation between the non-primate FIV lentivirus and HIV-1 at early and late events of retroviral replication.

Studies with chimeric EIAV/HIV and MLV/HIV vectors did not reveal a similar degree of functional conservation with HIV-1. Although HIV-1 structural and enzymatic proteins could mediate reverse transcription, stable transduction was absent; however, each of the EIAV/HIV and MLV/HIV chimeric vectors exhibited distinct episomal profiles (Figs. 41, 42, & 44). The episomal profiles were contingent upon the HIV-1 reverse transcriptase to recognize *cis* elements in the packaged chimeric vector RNA. Proper processing during reverse transcription is dependent upon two *cis* elements employed as primers to initiate negative strand (primer binding site [PBS]) and positive strand (3' polypurine tract [PPT]) synthesis (Fig. 40). A recent study demonstrated that the episomal profile is dependent upon

the capacity of the HIV-1 reverse transcriptase to recognize the 3' PPT (99). In the case of EIAV/HIV chimeric vector RNAs the HIV-1 reverse transcriptase could effectively recognize the EIAV PBS and 3' PPT to generate episomal products that are predominantly linear vector DNAs; therefore, replacing EIAV *att* sites with HIV-1 *att* sites were sufficient to enhance efficiency of stable transduction. This work is the first evidence for the capacity of HIV-1 to mediate reverse transcription of an EIAV-derived vector RNA (Fig. 41), as well as integration following *att* site replacement. Since HIV-1 reverse transcriptase can recognize the EIAV PBS and 3' PPT to generate linear vector DNAs competent for integration, HIV-1 may be more functionally related to EIAV than MLV.

Replacing the *att* sites for MLV/HIV chimeric vectors was not sufficient to recover integration, most likely since the dominant episomal form was 1-LTR vector DNAs, and not linear forms. Changing the PBS & 3' PPT, in addition to the *att* sites, dramatically improved integration (Fig. 43). Therefore, in the absence of a PBS & 3' PPT that can efficiently prime negative and positive strand synthesis for the HIV-1 RT, non-specific priming of reverse transcription may occur; thereby, altering the episomal profile. Similarly, previous results demonstrated that deletion of the HIV-1 3' PPT altered the episomal profile of a HIV-1 vector from preferentially linear substrates to 1-LTR circles (99). Other studies have also demonstrated that a cognate 3' PPT is required for proper removal of the PPT primer by the RNase H activity of RT to ensure formation of a correct U3 end for subsequent integration (32, 33). Regarding the PBS, as discussed in chapter 3 previous studies indicate that HIV-1 RT can utilize the MLV PBS at reduced efficiency (51); however, exchanging the MLV PBS with the HIV-1 PBS augmented stable transduction in the presence of HIV-1 *att* sites. Like the 3' PPT, a cognate PBS is critical for generating a correct U5 end for subsequent

integration (61, 159, 194, 213). In molecular studies presented here two different types of MLV/HIV chimeric vectors were utilized, one that only contained the HIV-1 RRE (MLV/HIV RRE, Figs. 43 & 44) and HIV-1 RRE + RU5PS (MLV/HIV RRE + RU5PS, Fig. 42). The MLV/HIV RRE + RU5PS vector also contained the HIV-1 R, U5, & PBS regions in the middle of the vector. In both cases Southern blot analysis using a 5' probe revealed a predominant 1-LTR circle form. A 3' probe revealed a dominant amount of varying sized linear forms present for the MLV/HIV RRE chimeric vector that were not integration competent (Fig. 44); however, although a 3' probe for the MLV/HIV RRE + RU5PS revealed the presence of linear episomes, the 1-LTR form remained the dominant form (Fig. 42). The insertion of the HIV-1 PBS could have introduced a dominant negative strand initiation site into the MLV vector that, along with the absence of a competent 3' PPT, may have contributed to dramatic increases of 1-LTR episomes. Packaging an MLV RNA containing the HIV-1 PBS could have enhanced the efficiency of intiating reverse transcription. This concept is in accordance with previous studies demonstrating the frequent use of a second PBS site artificially incorporated into a single retroviral RNA (208, 209). Nevertheless, experiments include altering the *att* sites, PBS, and 3' PPT in the context of the HIV-1 RRE + RU5PS may reveal further insights into the mechanism. Furthermore, the inherent generation of episomal vectors from heterologously packaged MLV/HIV and EIAV/HIV chimeric vector RNAs provides a safe, and innovative, system for the production of nonintegrating vectors to deliver therapeutic transgenes.

The heterologous MLV/HIV RRE + RU5PS RNA packaging system yields transduction efficiencies that approach those of standard HIV-1 vectors (chapter 3). The unique capacity of the heterologous vector to remain episomal exposes it to manipulation for

therapeutic gene delivery purposes. Episomal vectors have recently been sought as safer alternatives to integrating lentiviral vectors for gene therapy protocols requiring transient gene expression in dividing cells, or long-term expression in non-dividing cells (11, 210). HIV-1 delivery of the chimeric heterologous vector resulted in the dominant formation of 1-LTR episomal forms (Fig. 42), which was indicative of alterations in reverse transcription. Gene expression from the chimeric MLV/HIV system remains a challenge, yet, like non-integrating HIV-1 vectors (6, 14, 99, 137, 173, 179, 207, 219), expression is detectable following *in vivo* transduction of neurons in the mouse and rat brain striatum (Figs. 34-36). Restricting the synthesis of linear episomes through the use of the chimeric MLV/HIV vector system may impart improved safety benefits, over conventional non-integrating HIV-1 vectors, by reducing illegitimate integration.

The chimeric MLV/HIV vector packaged into HIV-1 viral particles curtails the presence of linear episomal forms that may be substrates for illegitimate integration as described earlier by Kantor et al. (99), as well as nonhomologous integration at sites of strand breakage in the host cell genome. A non-integrating vector that minimizes perturbations of the host cell genome would be most desirable for gene therapy protocols. Heterologously packaged vectors can also serve as a model system to investigate retroviral *cis* elements and *trans* factors that influence the transduction process. This is exemplified by the distinct transduction profiles of differently packaged chimeric vectors, which provided functional evidence demonstrating that the HIV-1 transduction mechanism is most closely related to FIV (FIV *cis* elements are sufficient for HIV-1 to mediate reverse transcription and integration), followed by EIAV (EIAV *cis* elements sufficient for HIV-1 mediated reverse

transcription, not integration), and MLV (MLV *cis* elements not amenable to HIV-1 mediated reverse transcription or integration).

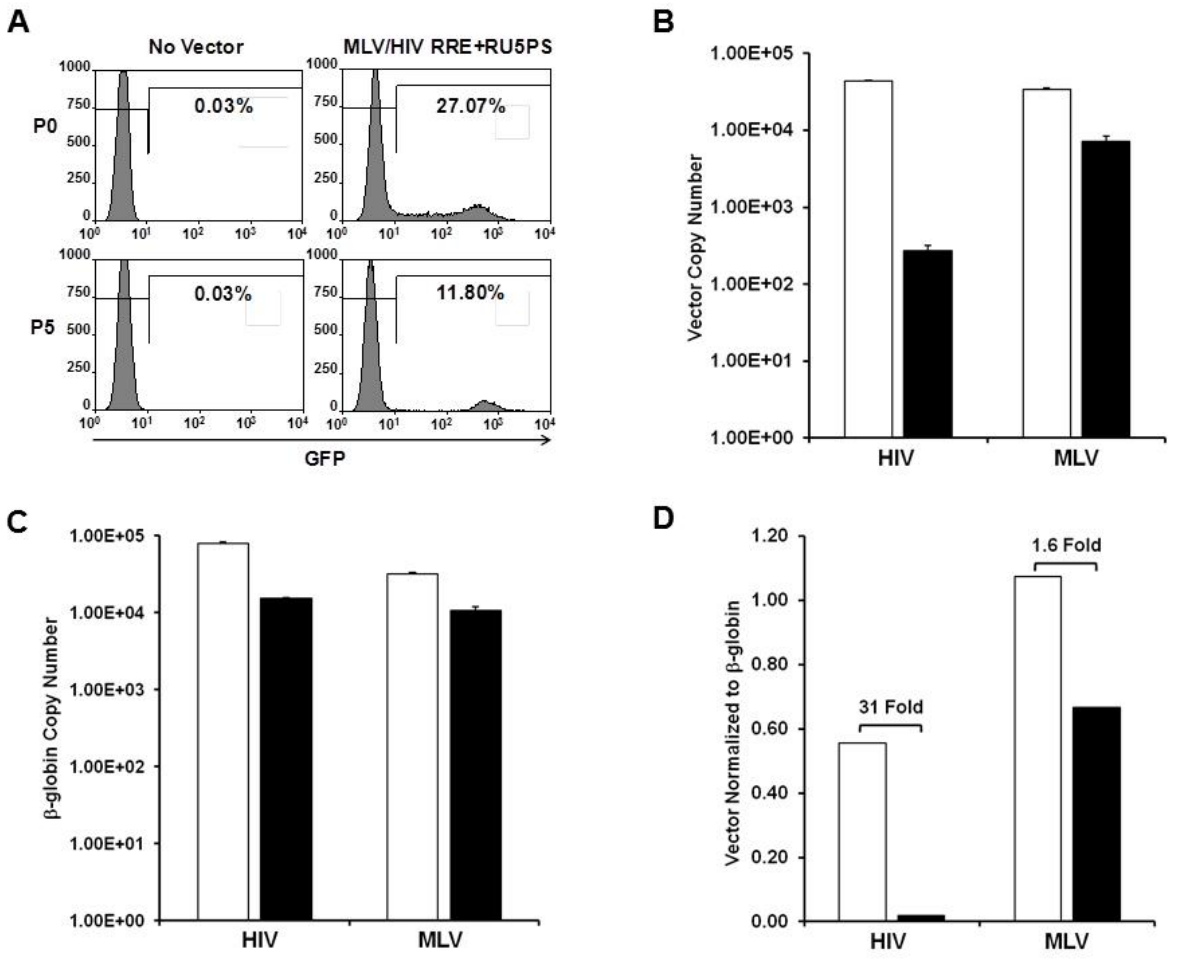


Figure 32. Heterologous MLV/HIV RNAs do not integrate following delivery with HIV-1 viral particles. A. Transduction of 293T cells with MLV/HIV RRE+RU5PS at 5 days post-transduction (no passaging of cells, P0), and after 5 passages of cells (P5). Percent GFP positive cells were assessed by FACscan analysis and compared to non-transduced (No Vector) 293T cells. B-D. MLV/HIV RRE+RU5PS chimeric vector was packaged into HIV-1 and MLV viral particles in the presence of Rev. 293T cells were transduced with equivalent transducing units for HIV-1 and MLV packaged vectors. Total cellular DNA was harvested at 5 days post-transduction (episomal and integrated vector DNA, P0), and after five cell passages (integrated vector DNA, P5). Vector DNA copy number as measured by qPCR to the WPRE **(B)**, and β-globin DNA copy number **(C)**, were determined by qPCR. Data are shown without cell passages (P0; white bars) and after 5 cell passages (P5; black bars). Vector DNA copy number was normalized to β-globin copy number **(D)**, and fold decreases in vector DNA levels, after passaging cells, are shown. Error for all bar graphs is expressed as ±S.D.

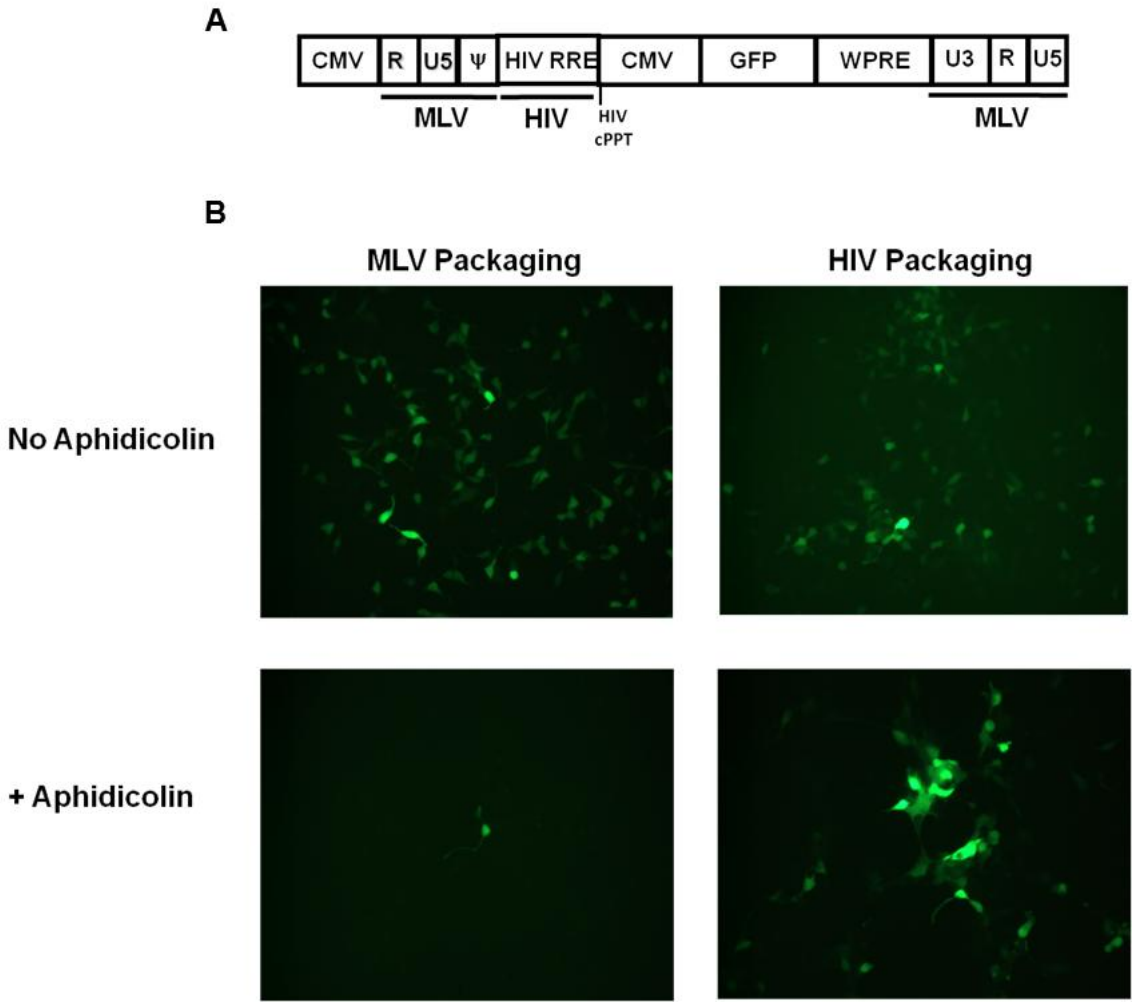


Figure 33. ***In vitro*** **delivery of MLV/HIV chimeric vector by HIV-1 viral particles to non-dividing 293T cells. A.** An early generation chimeric MLV/HIV RRE vector, similar to that utilized in chapters 2 & 3, except that the firefly luciferase gene was not present. **B.** The chimeric vector in part A was packaged into MLV or HIV-1 viral particles for delivery to 293T cells that were arrested at the G1/S phase of the cell cycle with 5 μg/ml aphidicolin.

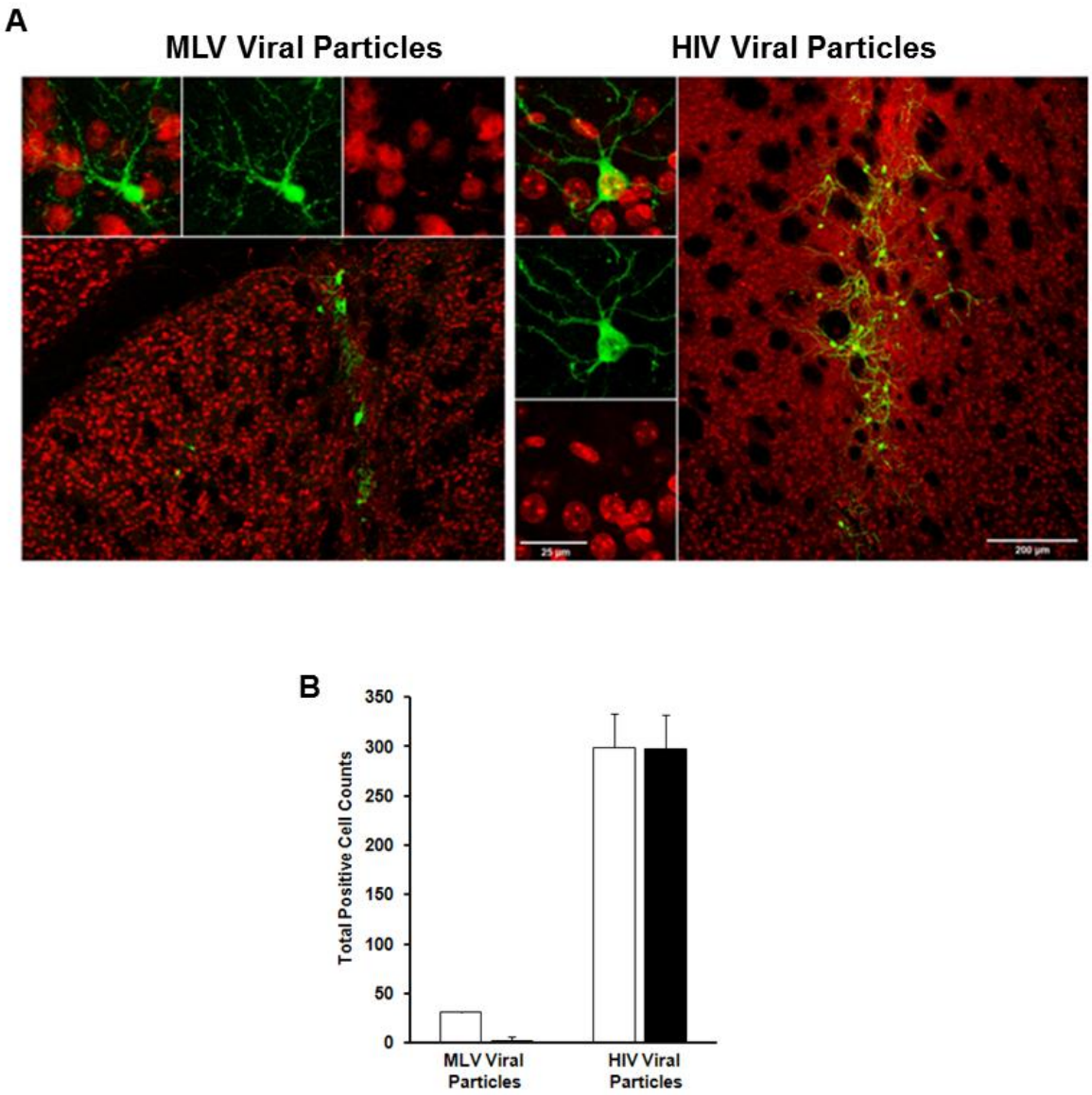


Figure 34. HIV-1 structural and enzymatic proteins can deliver a heterologously packaged MLV/HIV chimeric RNA to non-dividing cells in mouse brain striatum. A. Mouse brains were injected into the striatum with equivalent transducing units of MLV/HIV RRE vector packaged into either MLV (left panels), or HIV-1 (right panels), derived viral particles. Brain sections were imaged by confocal microscopy following co-staining for neurons (NeuN, red) and vector particles (GFP, green). Images depicting both vector and neurons can be seen for MLV (top left and low magnification) and HIV (left top and low magnification) viral particles. Independent images of vector and neurons are shown for MLV (top middle and top right, respectively) and HIV (left middle and left bottom, respectively) viral particles. **B.** The graph represents the total number of cells scored for GFP only (white bars), and colocalized GFP + NeuN (black bars) from 5 mice in each group. Error for bar graph is expressed as ±S.D.

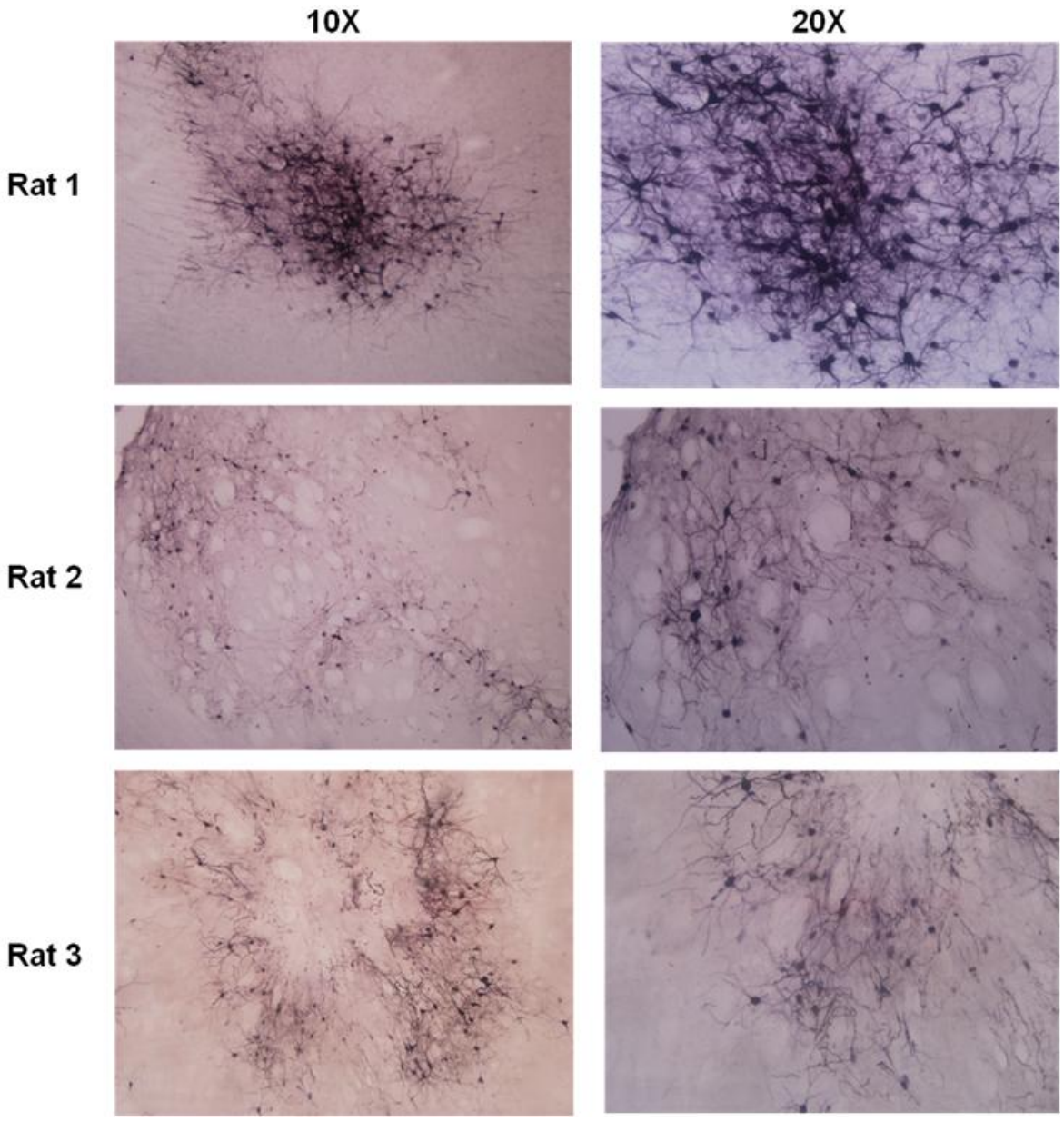


Figure 35. HIV-1 structural and enzymatic proteins can deliver a heterologously packaged MLV/HIV chimeric RNA to non-dividing cells in rat brain striatum. Rat brains were injected bilaterally into the striatum with equivalent transducing units of MLV/HIV RRE vector packaged into HIV-1 derived viral particles. Brain sections were imaged on a slide analyzer following detection of vector derived GFP expression from transduced cells by anti-GFP-biotinylated antibody, and subsequent secondary sensitive to DAB staining. The images depict the extent of neuronal transduction in the rat brain striatum, from three independent rat brains, shown at 10X and 20X magnifications.

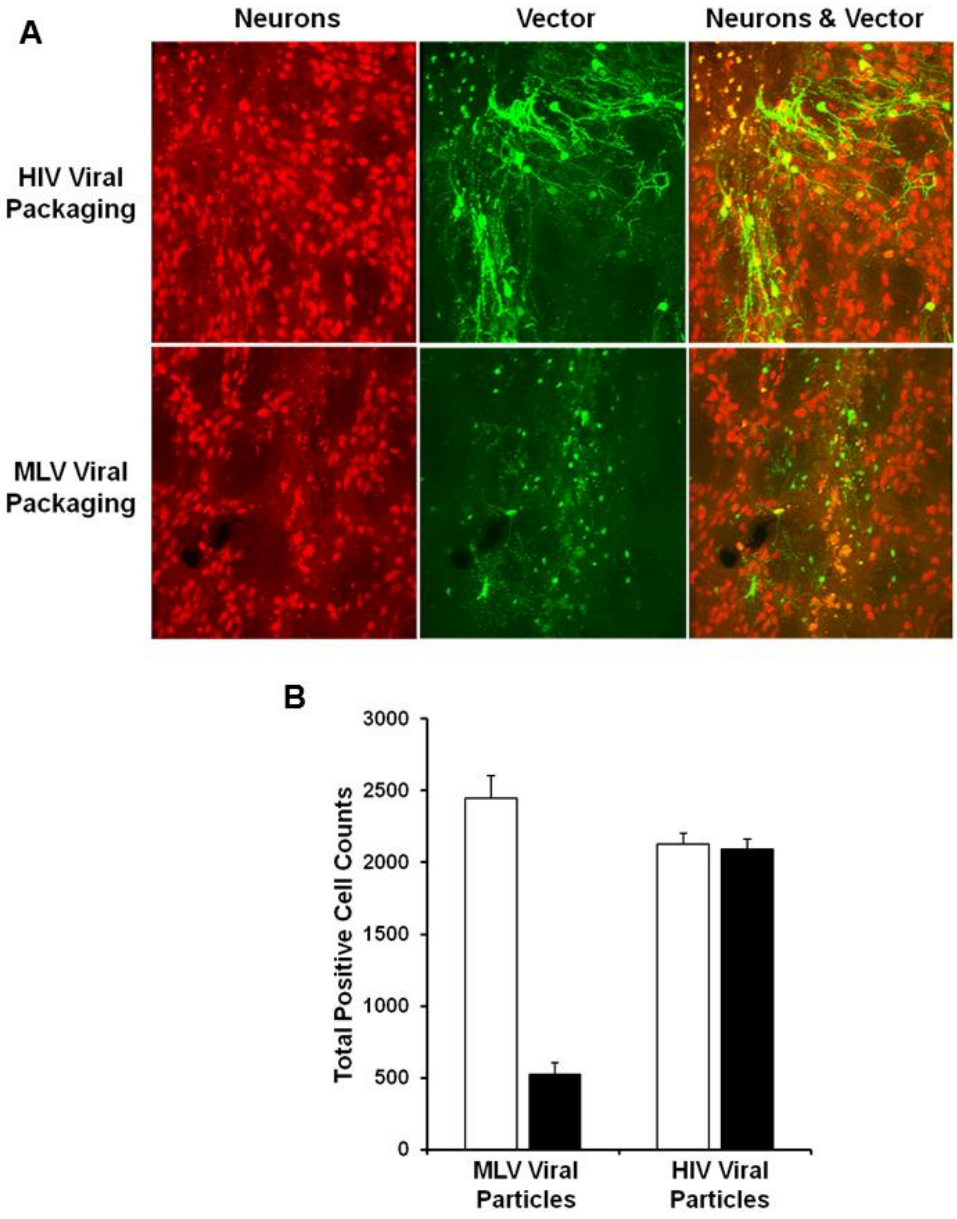


Figure 36. HIV-1 structural and enzymatic proteins can deliver a heterologously packaged MLV/HIV chimeric RNA to non-dividing cells in rat brain striatum. A. Rat brains were injected into the striatum with equivalent transducing units of MLV/HIV RRE vector packaged into either MLV (bottom panels), or HIV-1 (top panels), derived viral particles. Brain sections were imaged by confocal microscopy following co-staining for neurons (NeuN, red) and vector particles (GFP, green). Images depicting both vector and neurons can be seen for MLV (bottom, far right) and HIV (top, far right) viral particles. Independent images of vector and neurons are shown for MLV (bottom, left & middle) and HIV (top, left & middle) viral particles. **B.** The graph represents the total number of cells scored for GFP only (white bars), and colocalized GFP + NeuN (black bars) from 6 rats in each group. Error for bar graph is expressed as ±S.D.

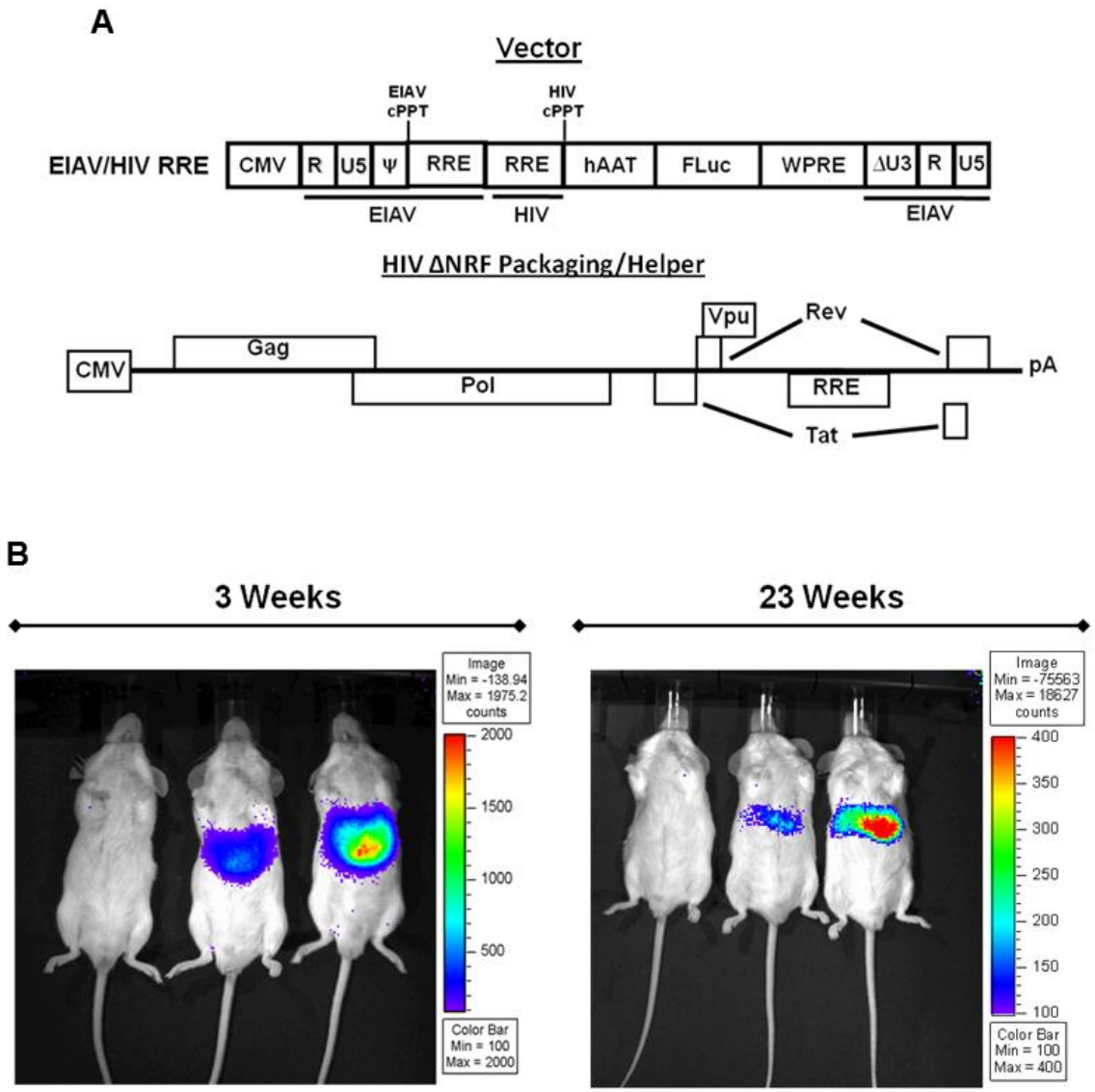


Figure 37. HIV-1 structural and enzymatic proteins can deliver a heterologously packaged EIAV/HIV RRE chimeric vector RNA to mouse liver. A. The EIAV/HIV RRE vector contains a liver specific promoter (human alpha anti-trypsin promoter) driving expression of the firefly luciferase gene. Vector RNAs were packaged into HIV-1 viral particles, derived from ΔNRF packaging construct, and produced in 293T cells. **B.** Concentrated vector was delivered intraperitoneally to two separate animals (middle and right), and compared to a PBS control (left). Mice were imaged at 3 weeks and 23 weeks post-injection. Scale at 23 weeks (400 max) represents a 5 fold increase in sensitivity compared to scale shown at 3 weeks (2000 max).

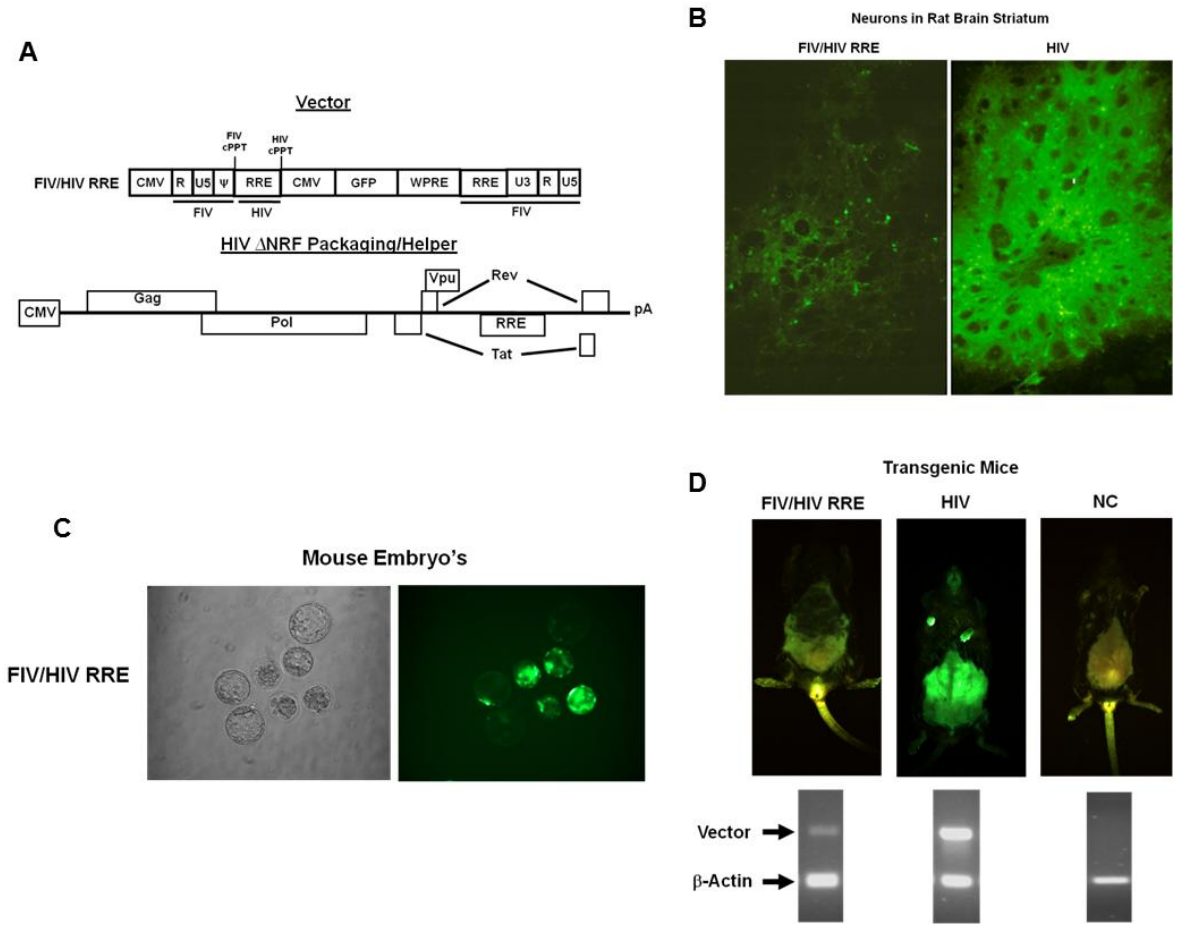


Figure 38. HIV-1 structural and enzymatic proteins can stably deliver a heterologously packaged FIV/HIV RRE chimeric vector RNA to mouse brain striatum and mouse embryos for generation of transgenic mice. A. Heterologous FIV/HIV RRE vector RNAs were packaged into ΔNRF derived HIV-1 viral particles, produced in 293T cells. **B.** Concentrated chimeric viral particles were administered to rat brain striatum. GFP expression from the vector was captured by fluorescence microscopy. **C.** Single cell mouse embryos were administered concentrated chimeric vector particles into the perivitelline space. GFP expression was captured by fluorescence microscopy. **D.** Transgenic mice were generated from tranduced embryos implanted into pseudopregnant females. GFP expression could be captured after removal of hair from mouse abdomens. Transgenic animals were confirmed by standard PCR for vector and β-actin on gDNA, followed by ethidium bromide detection in an agarose gel.

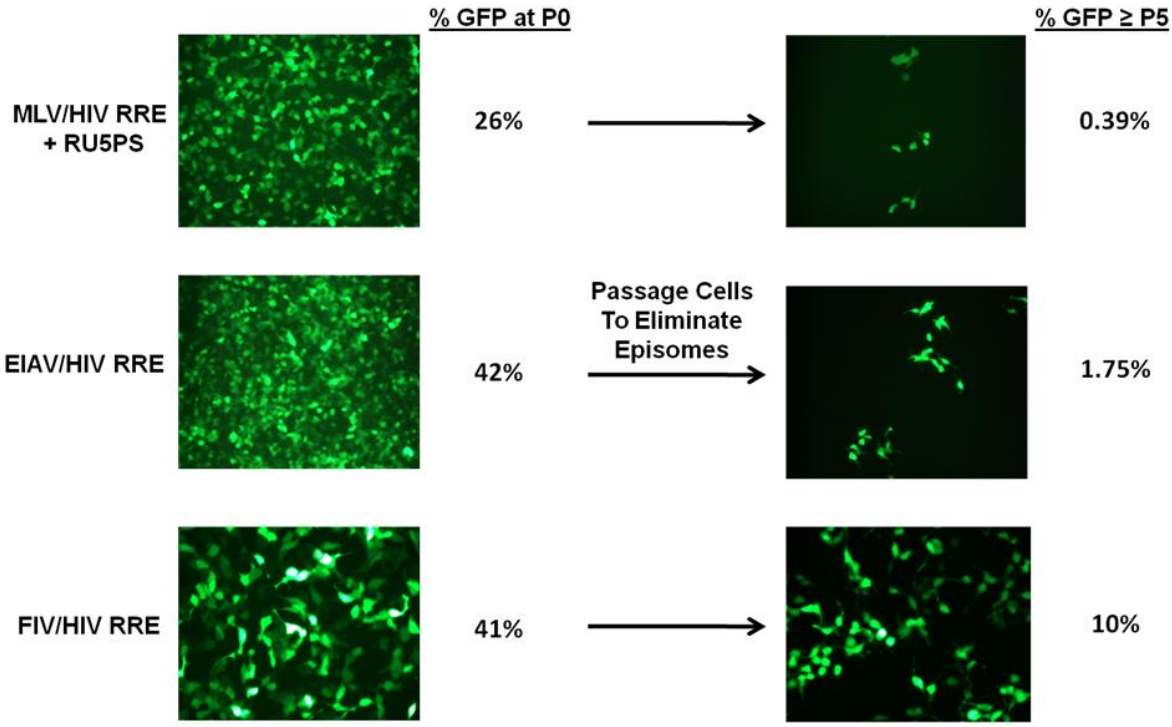


Figure 39. HIV-1 structural and enzymatic proteins deliver chimeric vector RNAs with distinct transduction profiles. Chimeric vector particles were generated in 293T cells with each of the indicated vectors packaged into HIV-1 viral particles. 293T cells were transduced with the chimeric vector particles and GFP expression was detected at 5 days post-transduction (P0) or after at least 5 passages of the cells (≥P5) to eliminate episomal cDNA forms. GFP expression was detected by fluorescence microscopy and FACScan analysis. The percent GFP positive cells are indicated before and after passaging cells.

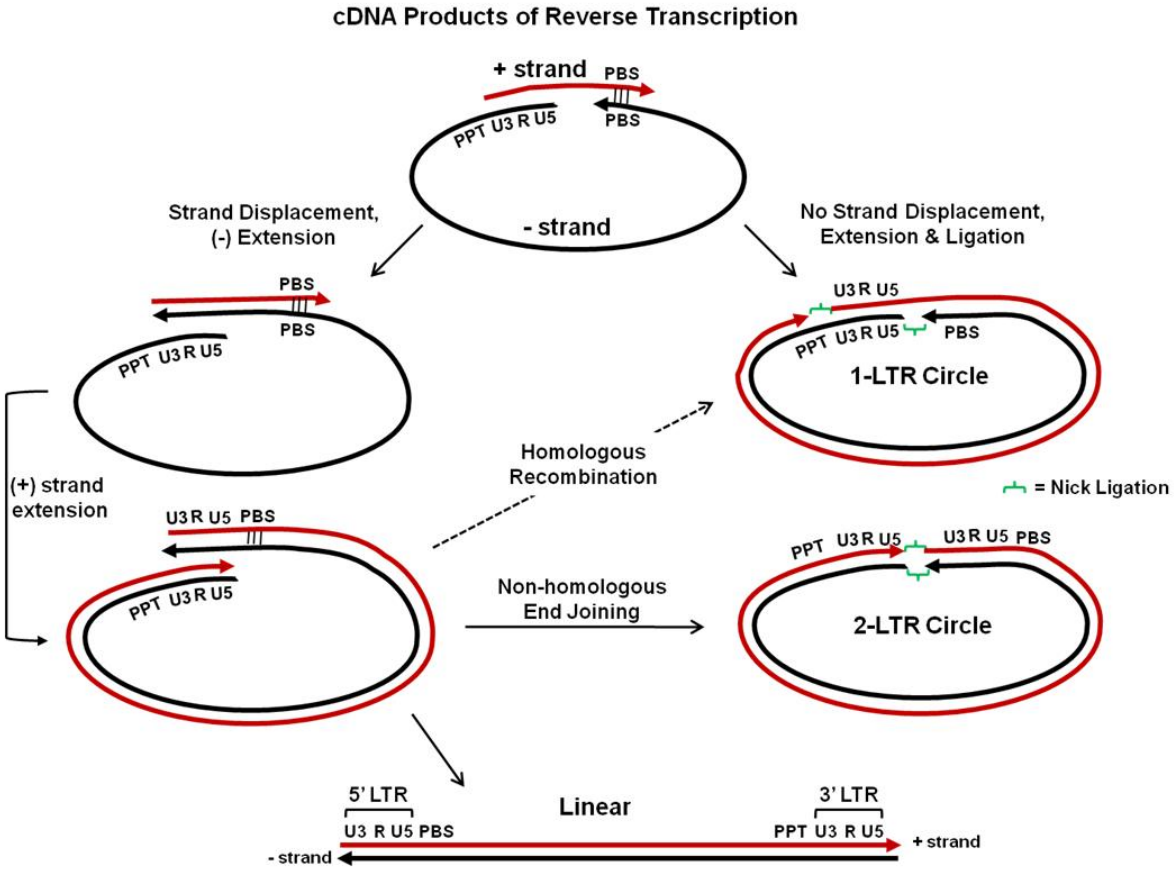


Figure 40. DNA products of retroviral reverse transcription. The diagram begins depicting a nearly complete minus (-) strand (black line), which was initiated by the $tRNA^{Lys3}$ priming reverse transcription from the primer binding site (PBS). The plus (+) strand (red line) synthesis was initiated by priming from the 3' polypurine tract (PPT). Under conventional circumstances (-) strand synthesis is completed through displacement of the (+) strand, and extension using the (+) strand as template for completion. Concurrently, (+) strand is completed using the (-) strand as template to generate a linear form that is competent for processing and integration mediated by integrase. In addition, 1-LTR circles are proposed to be generated from homologous recombination between the two LTRs, or more relevant here in the absence of strand displacement there may be complete extension by the reverse transcriptase and ligation of the nicks (indicated in green) at the ends of the LTRs. Lastly, the 2-LTR episomal form is commonly generated from linear molecules through non-homologous end joining mediated by host cell proteins.

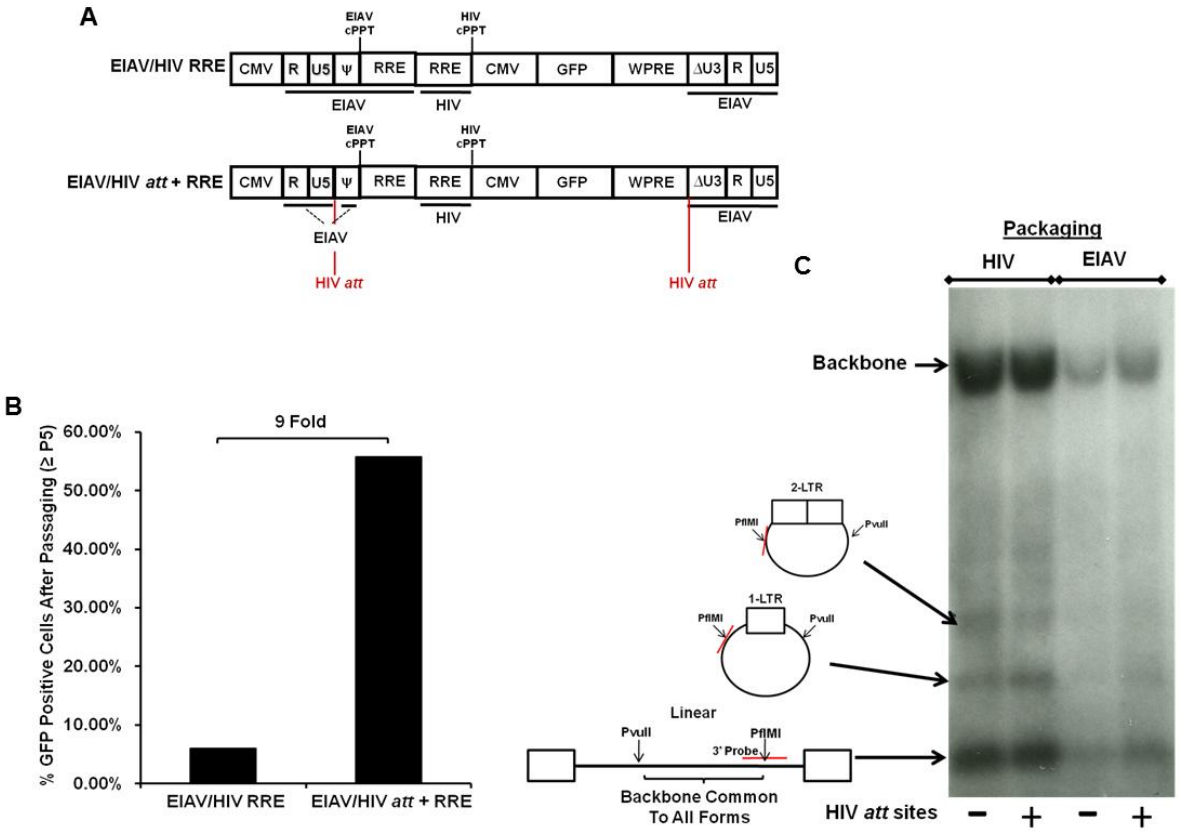


Figure 41. HIV-1 *att* sites augment stable transduction of EIAV/HIV RRE chimeric vector. A. The EIAV *att* sites in the chimeric EIAV/HIV RRE vector were altered to those of HIV-1 *att* sites (red). The vector RNAs were packaged into HIV-1, or EIAV, derived viral particles produced in 293T cells. **B.** 293T cells were transduced with equivalent GFP transducting units of EIAV/HIV RRE, and EIAV/HIV *att* + RRE vectors. Stable integration was examined by FACScan analysis for GFP expression after ≥5 passages of transduced cells to eliminate episomal forms. **C.** Southern blot analysis of total DNA purified from 293T cells transduced with EIAV/HIV RRE (- *att* sites) or EIAV/HIV *att* + RRE (+ *att* sites) chimeric vectors packaged into HIV-1 or EIAV viral particles. Episomal products were differentially detected with a 3' probe following digestion with PvuII + PflMI. The backbone is common to all forms between PvuII and PflMI, whereas 2-LTR, 1-LTR, and linear forms can be distinguished by size separation.

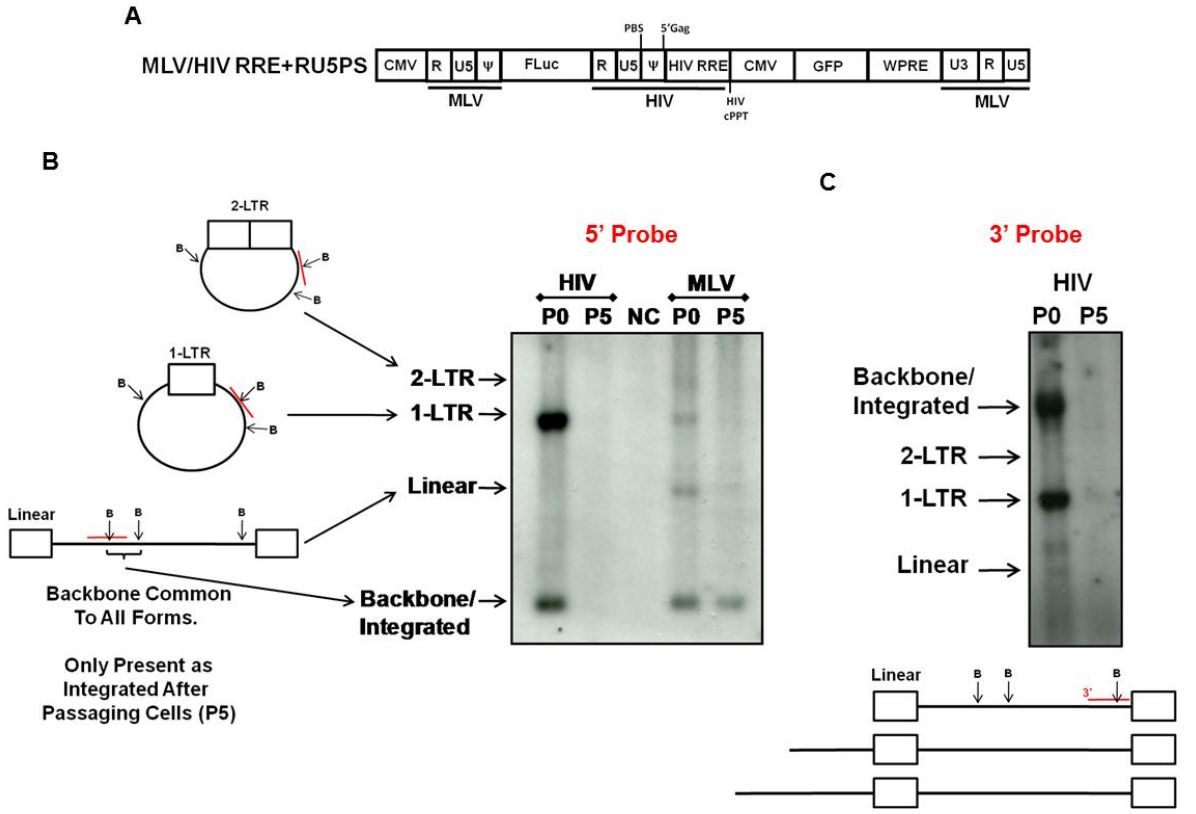


Figure 42. Heterologous MLV RNAs form, predominantly, 1-LTR episomal cDNAs following delivery with HIV-1 viral particles. A. MLV/HIV RRE+RU5PS chimeric vector was packaged into HIV-1 and MLV viral particles in the presence of Rev. **B & C.** 293T cells were transduced with equivalent transducing units for HIV-1 and MLV packaged vectors. Total cellular DNA was harvested at 5 days post-transduction (episomal and integrated vector DNA, P0), and after five cell passages (integrated vector DNA, P5). Southern blot analysis of total DNA isolated from 293T cells transduced with MLV/HIV RRE+RU5PS vector packaged into either HIV, or MLV, viral particles (as indicated above lanes). DNA was digested with BsrGI (B) to distinguish between 2-LTR, 1-LTR, and linear episomal forms, as well as the vector backbone which is indicative of integrated vector DNA after passaging cells. Independent blots were probed with a 5' probe **(B),** or 3' probe **(C)**. The 3' probe may reveal linear forms not detectable by the 5' probe.

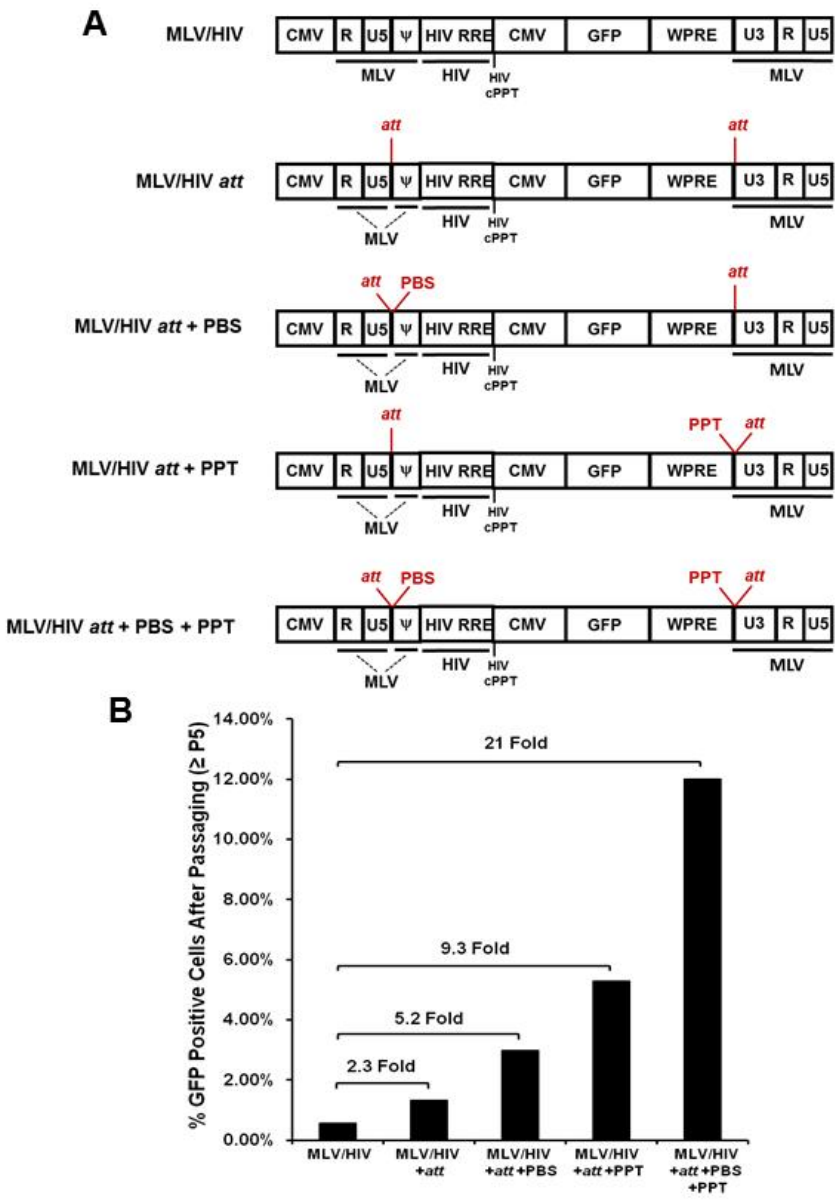


Figure 43. Stable transduction of a chimeric MLV/HIV vector requires HIV-1 *cis* elements that prime reverse transcription and facilitate integration. A. The MLV *att* sites, PBS, and PPT in the chimeric MLV/HIV RRE vector were altered to those of HIV-1 *att* sites, PBS, and PPT (red) where indicated. The vector names indicate the HIV-1 *cis* elements incorporated. The vector RNAs were packaged into HIV-1 derived viral particles produced in 293T cells. **B.** 293T cells were transduced with equivalent GFP transducing units of each chimeric MLV/HIV vector. Stable integration was examined by FACScan analysis for GFP expression after ≥5 passages of transduced cells to dilute episomal forms. Stable transduction is expressed as the percent GFP positive cells after passaging transduced cells, which was determined as a ratio relative to the GFP positive cells prior to passaging. Fold increases in stable transduction, relative to the MLV/HIV chimeric vector containing MLV *att* sites, PBS, and PPT.

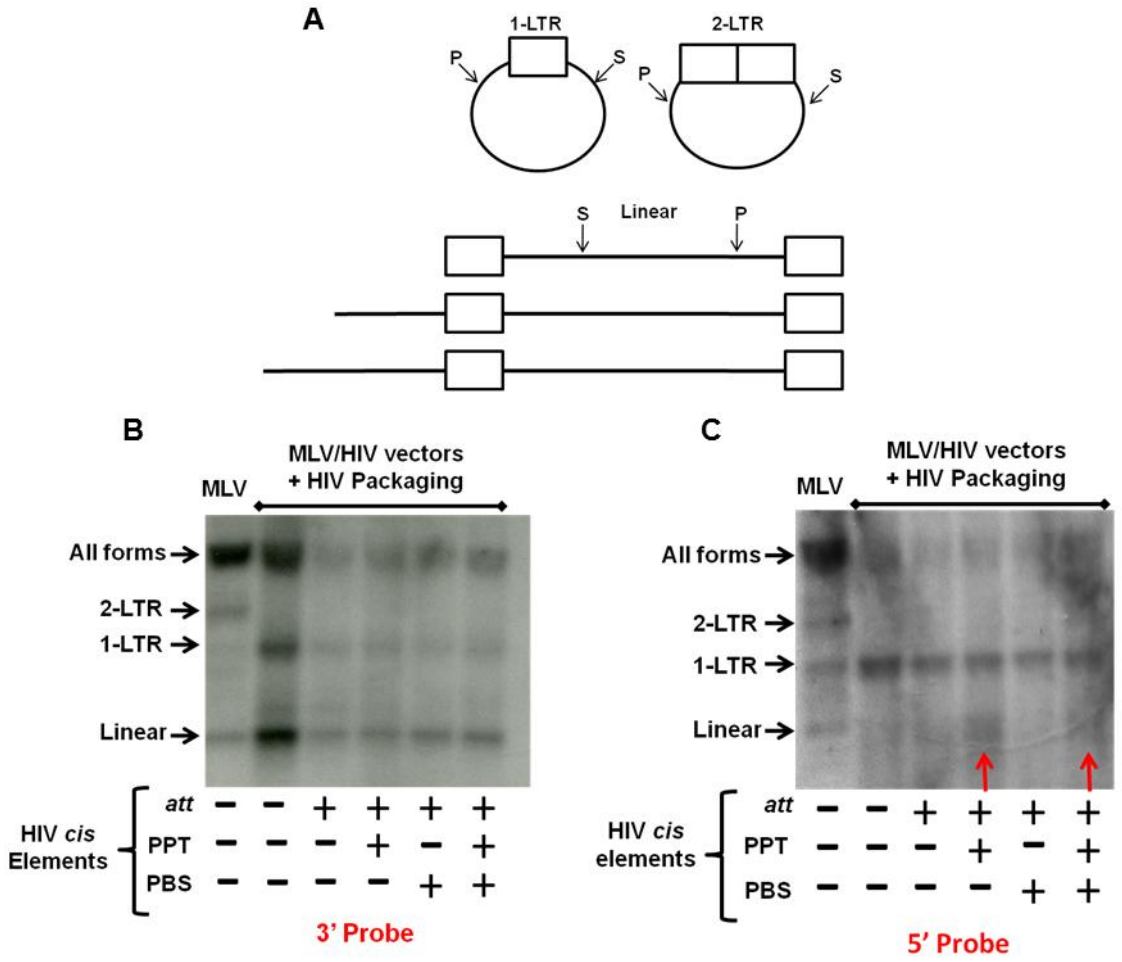


Figure 44. Altering PBS and PPT *cis* elements in chimeric MLV/HIV vectors changes the episomal profile. A. Episomal products of MLV/HIV chimeric vectors (Fig. 43) were differentially detected with a 3' probe **(B)**, or a 5' probe **(C)**, following digestion with SexAI (S) and PflMI (P). The backbone is common to all forms between SexAI and PflMI, whereas 2-LTR, 1-LTR, and linear forms can be distinguished by size separation. **B & C.** 293T cells were transduced with equivalent transducing units for HIV-1 and MLV packaged vectors. Total cellular DNA was harvested at 5 days post-transduction (episomal and integrated vector DNA, P0). Southern blot analysis of total DNA isolated from 293T cells transduced with chimeric MLV/HIV vectors packaged into either HIV, or MLV, viral particles (as indicated above lanes). Independent blots were probed with a 3' probe **(B),** or 5' probe **(C)**. The 3' probe may reveal linear forms not detectable by the 5' probe. However, the 5' probe can reveal increases in linear forms competent for integration. Different MLV/HIV chimeric vectors are indicated by the HIV-1 *cis* elements incorporated (*att* sites, PPT, or PBS) below each Southern blot.

Chapter 5

CONCLUSIONS AND FUTURE DIRECTIONS

From the perspective of viral pathogenesis, the viral replication process is typically divided into two stages early events of infection for the virus to enter a host cell, and late events whereby viral RNAs and proteins are expressed, and employed to generate nascent viral particles. This makes sense since this order of events will occur in the same host cell. However, an alternative perspective could be that the RNA and proteins incorporated into newly forming retroviral particles may govern subsequent infection events within a host cell. Therefore, one might consider early events of viral replication to engender mechanisms that lead to viral particle assembly for HIV-1, as well as other viruses, and how particle assembly could influence early infection events within naive host cells. The organization of the research in this dissertation reflects the latter sequence of events. The research presented in chapters 2 & 3 reveal a central contribution of the Rev/RRE system to the process of efficiently and specifically encapsidating RNAs into HIV-1 derived viral particles. The conventional canonical packaging signal, ψ, is the dominant *cis* packaging signal recognized by the HIV-1 Gag polyprotein; however, work presented in chapter 3 demonstrates that efficient RNA encapsidation also requires *cis* elements in the 5' UTR (R, U5, & PBS) that are upstream of the canonical packaging signal. Still, most notable was that the entire 5' UTR element lacked the capacity to enhance encapsidation of heterologous RNAs. Only in the presence of the Rev/RRE system could the 5' UTR *cis* elements dramatically increase RNA encapsidation efficiency into HIV-1viral particles (chapter 3). In contrast, the Rev/RRE

system could augment encapsidation efficiency of heterologous RNAs into HIV-1 viral particles in the absence of a canonical HIV-1 packaging signal (chapter 2). The Rev/RRE system positively influenced encapsidation of heterologous RNAs derived from three different retroviruses (MLV, FIV, & EIAV) (chapter 2); an indication that this mechanism was not dependent on a particular RNA context. Interestingly, each of these encapsidated heterologous RNAs exhibited distinct transduction mechanisms, which were dependent on the capacity of HIV-1 reverse transcriptase and integrase proteins to recognize *cis* elements in the heterologous RNAs/vector DNAs (chapter 4). Thus, returning to the discussion on early and late events of replication, in these studies the heterologous RNAs encapsidated into the HIV-1 viral particles determined the transduction profile in subsequent host cells. Moreover, these unique heterologous packaging systems may be manipulated for therapeutic gene delivery protocols.

HIV-1 Rev Augments Encapsidation of Heterologous RNAs into HIV-1 Viral Particles, Independent of the Canonical Packaging Signal.

In the absence of a HIV-1 canonical *cis* packaging signal, or any *cis* elements from the 5' UTR, the HIV-1 Rev/RRE can mediate an increase in heterologous RNA encapsidated into a HIV-1 viral particle (chapter 2, Fig. 13 & 14). Moreover, this increase appears to be specific to RNAs harboring the RRE (chapter 2). Since HIV-1 Rev has not been demonstrated to be encapsidated into viral particles it might be expected that Rev does not mediate the direct incorporation of the RNA into viral particles. Taking this into account, a model for this mechanism might be that Rev influences the ability of RRE-containing RNAs to concentrate in cytoplasmic locations that make the RNA amenable to interactions with the

HIV-1 Gag polyprotein. Even if this were the case, however, the absence of the major HIV-1 *cis* packaging element raises the question of how the nucleocapsid protein, within the context of the Gag polyprotein, could interact with the heterologous RNA for subsequent encapsidation/assembly of viral particles at the host cell plasma membrane. In the absence of a canonical packaging signal the nucleocapsid and matrix proteins, in the context of the Gag polyprotein, were shown to interact non-specifically with viral RNAs (165, 166). Therefore, in the absence of a canonical packaging signal (such as with heterologous RNAs utilized in chapter 2) the Gag polyprotein may interact non-specifically with available RNAs. Under these circumstances specificity would initially be conferred by the Rev/RRE interaction (the Rev/RRE system conferred specificity onto RRE containing RNAs, chapter 2), and the capacity of the Rev-RRE interaction to locate RNAs to cytoplamic sites that also harbor HIV-1 Gag polyprotein. Although a non-specific interaction, the Gag polyprotein could then mediate transport to sites of particle assembly at the plasma membrane. This model is in accordance with a series of recent studies indicating that monomer/dimer Gag molecules interact with HIV-1 viral RNA genomes in the cytoplasm (116); and, employing highly sensitive techniques to examine single particle assembly, they demonstrate that Gag-RNA complexes are recruited to sites of particle assembly at the plasma membrane, where further multimerization of Gag on the preformed Gag-RNA complexes generates nascent viral particles (96, 97). Future experiments to support this model might include examining the cytoplasmic colocalization of the heterologous RNAs with HIV-1 Rev and Gag polyprotein. Previous evidence indicates that HIV-1 Gag and genomic RNA colocalize at perinuclear/centrosomal regions in the cytoplasm (178). Conceivably, the Rev/RRE system could mediate transport from the nucleus to such sites in the cytoplasm.

Importantly, the Rev/RRE has been demonstrated to recruit a number of host cell proteins (41, 71, 199) that may also influence the proposed model. In at least one case, ablation of hRIP (human Rev interacting protein) function, a HIV-1 Rev cofactor, precludes HIV-1 replication and causes a perinuclear accumulation of viral RNA (223). Apparently, interfering with hRIP activity would preclude RNA packaging; however in this study hRIP also obviates viral particle production, making it difficult to distinguish between the two possibilities. In our heterologous packaging system viral particle production was Rev-independent so that only the influence of Rev on heterologous RNA encapsidation could be measured. Therefore, the heterologous packaging system could be used to isolate the impact of cellular proteins on RNA encapsidation into HIV-1 viral particles. Employing a dominant negative hRIP protein (223), or shRNA knockdown experiments to a number of identified host cell proteins that interact with the Rev/RRE (41, 71, 199), would be very useful in parsing the effects of host cellular proteins on RNA encapsidation into HIV-1 viral particles.

Studies presented in chapter 2 not only reveal the influence of the HIV-1 Rev/RRE system on encapsidation, but also the potential for a conserved mechanism with the Rev protein from the non-primate lentivirus, feline immunodeficiency virus (FIV). According to titers, which may reflect packaged RNA, the FIV Rev (FRev) functioned similar to the HIV Rev (HRev) to efficiently increase titers of FIV/HIV chimeric vector RNAs, packaged into HIV-1 viral particles. In contrast, Rev from equine infectious anemia virus (EIAV) could not augment titers of EIAV/HIV chimeric vector RNAs into HIV-1 viral particles, even though HIV-1 Rev could (chapter 2, Fig. 15). Apparently, ERev may commandeer a mechanism that is distinct from that of HRev and FRev. Of course future studies should clarify if this is indeed a difference in RNA encapsidation. Nonetheless, a previous study indicates that in

human cells HIV-1 and EIAV exhibit distinct assembly sites, and that these can be altered by changing the RNA trafficking pathway (93). Importantly, a follow-up study indicated that efficient HIV-1 assembly is dependent upon correct RNA trafficking, and correct RNA trafficking was dependent on the Rev/RRE system (94). Therefore, HRev and FRev may share a trafficking pathway, whereby the function is conserved in human cells. Presumably, ERev would utilize a different trafficking pathway. Apparently, the difference in trafficking does not stem from the use of the CRM1 nuclear export pathway since it was previously demonstrated that FRev and ERev also utilize the CRM1 nuclear export pathway (164); thus, the differences in trafficking would most likely be contingent upon trafficking in the cytoplasm. Also of note, since the HRev could augment encapsidation of the EIAV/HIV RRE chimeric vector RNA the difference is probably not a consequence of EIAV *cis* elements in the 5' UTR (chapter 2, Figs. 14 & 15), but rather differences associated with the Rev proteins. Future studies may include the use of colocalization studies with different Rev proteins, heterologous RNAs, and Gag to investigate distinct trafficking differences attributed to Rev. Exploiting the differences obtained with different Rev proteins will clearly be advantageous in deciphering the mechanism of the Rev protein in RNA encapsidation.

The dominant function attributed to the Rev protein is nuclear export of intron-containing HIV-1 RNAs through the CRM1-dependent pathway (58, 59, 155, 163). Nuclear export is part of the RNA localization process, therefore may contribute to the fate of RNA in the cytoplasm. Manipulating viral RNA *cis* elements and *trans* factors that impact nuclear export have also been shown to affect viral particle assembly (93, 94, 149, 191, 201). As shown here altering the nuclear export pathway severely attenuated the effect of Rev on RNA encapsidation (chapter 2, Fig. 19), but did not abolish it; indicating that the pathway of

nuclear export may not absolutely determine the cytoplasmic fate of the RNA. More important may be trafficking of the Rev/RNA complex in the cytoplasm to sites where the Gag polyprotein can efficiently interact with the canonical packaging signal in the RNA.

An Efficient and Specific HIV-1 RNA Packaging System Requires the Concerted Effort of the Rev/RRE system and *Cis* Elements in the 5' UTR.

The HIV-1 Rev/RRE system can clearly augment encapsidation independent of *cis* elements in the 5' UTR; however, the efficiency of encapsidating a heterologous RNA is diminished compared to a construct with the Rev/RRE system and the entire complement of *cis* elements from the 5' UTR (chapter 3, Fig. 24). Previous studies indicate that the Rev/RRE can contribute to RNA encapsidation when the RRE is in the same RNA context as *cis* elements from the HIV-1 5' UTR (5, 19, 67, 181). These studies, however, did not distinguish the encapsidation contribution of the individual HIV-1 components to the collective influence of all the components together. The gain-of-function studies presented in this work demonstrate for the first time that *cis* elements in the 5' UTR (including the canonical packaging signal) require the Rev/RRE system to achieve efficient encapsidation (chapter 3, Fig. 24). Since the canonical packaging signal is the major *cis* element recognized by the Gag polyprotein, it might have been anticipated that in the presence of the Rev/RRE system the canonical packaging signal would be sufficient to achieve efficient encapsidation. However, independent incorporation of ψ (canonical packaging signal) in the context of the Rev/RRE system did not exhibit a gain in encapsidation (chapter 3, Fig. 25), which is in agreement with prior mutagenesis studies implicating *cis* deteminants in the R, U5, and PBS regions as important for efficient RNA encapsidation (37, 38, 81, 139). Data presented here

insinuate that the 5' UTR *cis* elements are not separable to achieve efficient encapsidation of heterologous RNAs (chapter 3, Fig. 25). Potentially, there is a requirement for long-range intramolecular interactions between *cis* elements in the R, U5, and PBS, with those in the canonical packaging signal, as previously indicated (50, 214). Exploiting the high resolution HIV-1 RNA structure, recently elucidated (Fig. 8) (212, 214), may be beneficial in generating specific mutations that disrupt identified long-range intramolecular RNA interactions, and assess the mutants for encapsidation efficiency in the context of our heterologous encapsidation system. Discerning how the Rev/RRE system influences specific and efficient recognition of *cis* elements in the 5' UTR by the Gag polyprotein will be an important focus of future studies. Apparently, RNA encapsidation into HIV-1 viral particles may be modeled as at least a two-step mechanism in which: i) the Rev/RRE system confers efficient and specific transport of the RNA from transcription in the nucleus to a particular location in the cytoplasm; and, ii) Gag interacts with the canonical packaging signal to transport the RNA from a cyoplasmic compartment to sites of particle assembly at the plasma membrane.

The requirement for the HIV-1 Rev/RRE system in the encapsidation mechanism implies that the Rev/RRE may confer specificity onto HIV-1 RNA throughout step 1 of the mechanism, from the time that Rev interacts with RNA in the nucleus. Such a mechanism might ensure early selection of viral RNA from the milieu of host cell RNAs, concomitant with transcription in the nucleus. In fact, results presented throughout chapters 2 & 3 demonstrate that this is not a general biological phenomenon, but rather specific to heterologous RNAs harboring the RRE *cis* element. Until now the specificity of the Rev/RRE system for RNA encapsidation has not been addressed. Importantly, the concerted

effect of the Rev/RRE system and 5' UTR *cis* elements observed for RNA encapsidation into HIV-1 viral particles was not possible into MLV derived viral particles (chapter 3, Fig. 30); consistent with the proposal that HIV-1 and MLV utilize distinct mechanisms to encapsidate viral RNA (53, 121).

There is an apparent requirement for packaging systems that can preferentially facilitate packaging of the viruses own genome to maintain viral fitness. Nonspecific packaging of cellular RNAs during generation of nascent HIV-1 viral particles has also been well documented (152, 162, 188). Data shown here demonstrate that the specificity conferred upon the vector RNA by the Rev/RRE system can mitigate the nonspecific packaging of producer cell RNAs (i.e. Rev-mediated increase packaging of HIV-1 vector RNA while concomitantly reducing nonspecific large T antigen RNA) (Fig. 31). Additionally, packaged cellular RNAs have been shown to be reverse transcribed, and recombine with the packaged viral RNA during reverse transcription in host cells (78, 123, 152). With regard to retroviral vector production, 293T cells were identified to be most amenable for this process through ectopic expression of vector particle components. Most notably 293T cells overexpress the large T antigen, which is well recognized to transform cells upon overexpression. Data presented here demonstrate that large T antigen RNA is readily packaged into HIV-1 derived viral particles and can be reverse transcribed in naïve host cells (Fig. 31); however, this concern can be eased by utilizing cells that do not overexpress large T antigen (Fig. 31). Although reverse transcription of large T antigen RNA indicates a potential to be stably introduced into a host cell genome, the frequency of this event may be extremely rare, therefore would require a highly sensitive assay for detection. Since this may have broad implication regarding the production of lentiviral vectors currently being used in clinical

trials (29, 30), considerable effort should be placed on developing a functional assay sensitive enough to detect stable transfer of large T antigen.

The research presented in chapters 2 & 3 reveal a central contribution of the Rev/RRE system to the process of efficiently and specifically encapsidating heterologous RNAs into HIV-1 derived viral particles. The results define a transferrable HIV-1 packaging system comprised of the Rev/RRE, 5' UTR *cis* elements, and Gag polyprotein. As mentioned above heterologous RNAs packaged during particle assembly in producer cells may influence events of transduction in subsequent host cells. Packaging three different heterologous RNAs (derived from MLV, EIAV, or FIV) into HIV-1 viral particles exposed distinct transduction properties.

Packaging heterologous RNAs into HIV-1 viral particles reveals unique transduction properties with applications for gene therapy.

The packaging of heterologous vector RNAs into HIV-1 derived viral particles is not only a model system for studying RNA encapsidation mechanisms, but has also been exploited to investigate retroviral *cis* elements and *trans* factors that influence the transduction process. HIV-1 structural and enzymatic proteins that comprise viral particles can mediate transduction of heterologously packaged RNAs derived from three different retroviral vectors: MLV, EIAV, and FIV. These vectors were able to be delivered to dividing and non-dividing cells *in vitro* and *in vivo* (chapter 4). A well established biological phenomenon is that MLV viral particles cannot transduce quiescent cells (217). This is the first demonstration that encapsidating a heterologous MLV vector RNA into HIV-1 viral particles facilitated transduction, which includes nuclear entry, of a chimeric MLV vector

RNA into non-dividing cells *in vitro* and *in vivo* (chapter 4 Figs. 33-36). These data demonstrate the capacity of HIV-1 structural and enzymatic proteins to confer nuclear import into quiescent cells upon a MLV derived RNA, and are in accordance with a study demonstrating that the HIV-1 capsid protein is critical for the transduction of non-dividing cells (218). The capacity to transduce arrested cells does not appear to be dependent upon *cis* elements in the vector, but rather proteins supplied by the HIV-1 viral particles. Although the HIV-1 cPPT (central polypurine tract) was included in our constructs during cloning, and it has been suggested to enhance transduction, more recent studies indicate that the previous list of HIV-1 karyophilic determinants (matrix, Vpr, integrase, & cPPT) are not required for transduction of nondividing cells (182, 216). Our heterologous packaging systems do not include Vpr, therefore in agreement with previous studies Vpr is not required for nuclear entry. The heterologous packaging system may further be beneficial in examining viral protein components that facilitate nuclear import through mutagenesis. Moreover, the *cis* elements and *trans* factors of the heterologous packaging system can be readily manipulated to investigate putative interactions, and the functional contribution of host cell proteins with different viral components to mediate transduction.

Of the three heterologous vector RNAs packaged into HIV-1 viral particles stable transduction was partially achievable with the chimeric FIV/HIV vector (chapter 4, Figs. 38 & 39), enough such that transgenic mice could be generated from single cell embryos (chapter 4, Fig. 38). These results were in line with those previously obtained for heterologous packaging and propagation of RNA derived from primate (184, 198), and the non-primate FIV (22) lentivirus's, into HIV-1 derived viral particles. As shown by functional studies with FIV Rev in chapter 2 and transduction results in chapter 4, there appears to be a

significant degree of functional conservation between FIV and HIV-1 that is not shared with EIAV and MLV.

In contrast to FIV/HIV chimeric vectors, the HIV-1 structural and enzymatic proteins did not mediate stable transduction of MLV/HIV and EIAV/HIV chimeric vectors. The MLV/HIV and EIAV/HIV chimeric vectors remained as episomes following transduction. Molecular analysis revealed that the episomal profiles of MLV/HIV and EIAV/HIV vectors were distinct. MLV/HIV vectors exhibited an abundance of 1-LTR episomes and linear forms that were not competent for integration (chapter 4, Fig. 42 & 44), whereas the episomal profile for chimeric EIAV/HIV vectors was comprised predominantly of linear forms (chapter 4, Fig. 41). Interestingly, stable transduction was recovered by correcting vector *cis* elements involved in distinct molecular processes. Regarding EIAV/HIV chimeric vectors replacing EIAV *att* sites with those of HIV-1 was sufficient (chapter 4, Fig. 41); however, MLV/HIV chimeric vectors required changing the *att* sites, as well as the PBS and PPT to those of HIV-1 (chapter 4, Fig. 43). Thus, altering EIAV *att* sites for recognition by HIV-1 integrase was minimally necessary for stable transduction of the EIAV/HIV chimeric vector; whereas, the MLV/HIV chimeric vectors required altering *cis* elements for both reverse transcription and integration. Clearly, these are the first studies that provide functional evidence demonstrating different degrees of functional conservation between retroviruses using a single viral packaging system. Episomal vectors that minimize perturbations of the host cell genome and remain stable in non-dividing cells would also be most desirable for therapeutic gene delivery protocols. The MLV/HIV and EIAV/HIV chimeric vectors can clearly transduce non-dividing/slowly dividing cells *in vivo*, and maintain transgene expression (chapter 4, Figs. 34-37); albeit, in the case of MLV/HIV

chimeric vectors the low level of GFP expression required the use of antibodies to increase detection sensitivity. Nonintegrating lentiviral vectors have recently been pursued as safer alternatives to integrating lentiviral vectors demonstrating transduction of the CNS, liver, muscle, and eye (6, 14, 99, 173, 179, 219). Despite the improved safety profile of episomes to diminish the potential for insertional mutagenesis, linear episomal products remain the dominant species, as shown for EIAV/HIV chimeric vectors, and have the potential to instigate genotoxicity through illegitimate integration (i.e. viral DNA integration that is not mediated by a functional integrase). Since the linear episomal species are the products of a successful reverse transcription process, it was recently realized that interfering with reverse transcription, through deletion of the 3' PPT, can alter the profile of episomal vector species to favor 1-LTR circles (99), thereby ameliorating the episome safety profile. A similar episomal profile is obtained with the MLV/HIV chimeric vectors packaged into HIV-1 viral particles (chapter 4, Fig. 42 & 44), indicative of its usefulness in therapeutic gene delivery protocols.

Significance

The heterologous RNA packaging system utilized here is an innovative universal packaging system that facilitates functional investigations of early and late events of HIV-1 viral replication. The studies presented in this dissertation demonstrate that a competent packaging system comprises the concerted effort of multiple HIV-1 *cis* elements and *trans* factors. As shown here the entire complement of *cis* elements from the 5' UTR were not sufficient to augment encapsidation of a heterologous RNA into HIV-1 viral particles. Importantly, these studies reveal that the Rev/RRE system is required for the 5' UTR *cis*

elements to positively influence RNA encapsidation. In fact, the data in this dissertation demonstrate that the Rev/RRE system can significantly augment encapsidation in the absence of all *cis* elements from the 5' UTR; albeit, the overall encapsidation efficiency is not comparable to heterologous vectors containing the 5' UTR and Rev/RRE system. As defined by the studies in this dissertation a HIV-1 packaging system, capable of conferring efficient and specific encapsidation of heterologous RNAs, should minimally comprise the Rev/RRE system, 5' UTR *cis* elements, and Gag polyprotein. These studies indicate that the encapsidation process may constitute at least a two-step mechanism in which: i) the Rev/RRE system confers efficient and specific transport of RNA from transcription in the nucleus to a particular location in the cytoplasm for subsequent Gag-RNA interaction; and, ii) Gag interacts with the canonical packaging signal to transport the RNA from a cyoplasmic compartment to sites of particle assembly at the plasma membrane. The same HIV-1 structural and enzymatic proteins that mediate encapsidation of heterologous RNAs also facilitate transduction of chimeric vector RNAs into nondividing cells *in vivo*. The dominant 1-LTR episomal profile of transduced MLV/HIV chimeric vector RNAs may have improved safety benefits over more conventional nonintegrating lentiviral vectors by reducing the potential for illegitimate perturbations of the host cell genome. These innovative vector systems may prove beneficial for therapeutic gene delivery to nondividing cells.

REFERENCES

1. **Abbink, T. E., and B. Berkhout.** 2008. HIV-1 reverse transcription initiation: a potential target for novel antivirals? Virus Res **134:**4-18.
2. **Abbink, T. E., M. Ooms, P. C. Haasnoot, and B. Berkhout.** 2005. The HIV-1 leader RNA conformational switch regulates RNA dimerization but does not regulate mRNA translation. Biochemistry **44:**9058-9066.
3. **Adam, M. A., and A. D. Miller.** 1988. Identification of a signal in a murine retrovirus that is sufficient for packaging of nonretroviral RNA into virions. J Virol **62:**3802-3806.
4. **Aldovini, A., and R. A. Young.** 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. J Virol **64:**1920-1926.
5. **Anson, D. S., and M. Fuller.** 2003. Rational development of a HIV-1 gene therapy vector. J Gene Med **5:**829-838.
6. **Apolonia, L., S. N. Waddington, C. Fernandes, N. J. Ward, G. Bouma, M. P. Blundell, A. J. Thrasher, M. K. Collins, and N. J. Philpott.** 2007. Stable gene transfer to muscle using non-integrating lentiviral vectors. Mol Ther **15:**1947-1954.
7. **Arnold, M., A. Nath, J. Hauber, and R. H. Kehlenbach.** 2006. Multiple importins function as nuclear transport receptors for the Rev protein of human immunodeficiency virus type 1. J Biol Chem **281:**20883-20890.

8. **Arrigo, S. J., and I. S. Chen.** 1991. Rev is necessary for translation but not cytoplasmic accumulation of HIV-1 vif, vpr, and env/vpu 2 RNAs. Genes Dev **5:**808-819.

9. **Arrigo, S. J., S. Heaphy, and J. K. Haines.** 1992. In vivo binding of wild-type and mutant human immunodeficiency virus type 1 Rev proteins: implications for function. J Virol **66:**5569-5575.

10. **Balvay, L., M. Lopez Lastra, B. Sargueil, J. L. Darlix, and T. Ohlmann.** 2007. Translational control of retroviruses. Nat Rev Microbiol **5:**128-140.

11. **Banasik, M. B., and P. B. McCray, Jr.** 2010. Integrase-defective lentiviral vectors: progress and applications. Gene Ther **17:**150-157.

12. **Banks, J. D., B. O. Kealoha, and M. L. Linial.** 1999. An Mpsi-containing heterologous RNA, but not env mRNA, is efficiently packaged into avian retroviral particles. J Virol **73:**8926-8933.

13. **Banks, J. D., A. Yeo, K. Green, F. Cepeda, and M. L. Linial.** 1998. A minimal avian retroviral packaging sequence has a complex structure. J Virol **72:**6190-6194.

14. **Bayer, M., B. Kantor, A. Cockrell, H. Ma, B. Zeithaml, X. Li, T. McCown, and T. Kafri.** 2008. A large U3 deletion causes increased in vivo expression from a nonintegrating lentiviral vector. Mol Ther **16:**1968-1976.

15. **Berkowitz, R. D., M. L. Hammarskjold, C. Helga-Maria, D. Rekosh, and S. P. Goff.** 1995. 5' regions of HIV-1 RNAs are not sufficient for encapsidation: implications for the HIV-1 packaging signal. Virology **212:**718-723.

16. **Berthold, E., and F. Maldarelli.** 1996. cis-acting elements in human immunodeficiency virus type 1 RNAs direct viral transcripts to distinct intranuclear locations. J Virol **70:**4667-4682.

17. **Bohne, J., and H. G. Krausslich.** 2004. Mutation of the major 5' splice site renders a CMV-driven HIV-1 proviral clone Tat-dependent: connections between transcription and splicing. FEBS Lett **563:**113-118.

18. **Bolinger, C., and K. Boris-Lawrie.** 2009. Mechanisms employed by retroviruses to exploit host factors for translational control of a complicated proteome. Retrovirology **6:**8.

19. **Brandt, S., M. Blissenbach, B. Grewe, R. Konietzny, T. Grunwald, and K. Uberla.** 2007. Rev proteins of human and simian immunodeficiency virus enhance RNA encapsidation. PLoS Pathog **3:**e54.

20. **Bray, M., S. Prasad, J. W. Dubay, E. Hunter, K. T. Jeang, D. Rekosh, and M. L. Hammarskjold.** 1994. A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. Proc Natl Acad Sci U S A **91:**1256-1260.

21. **Browning, M. T., F. Mustafa, R. D. Schmidt, K. A. Lew, and T. A. Rizvi.** 2003. Delineation of sequences important for efficient packaging of feline immunodeficiency virus RNA. J Gen Virol **84:**621-627.

22. **Browning, M. T., R. D. Schmidt, K. A. Lew, and T. A. Rizvi.** 2001. Primate and feline lentivirus vector RNA packaging and propagation by heterologous lentivirus virions. J Virol **75:**5129-5140.

23. **Brussel, A., D. Mathez, S. Broche-Pierre, R. Lancar, T. Calvez, P. Sonigo, and J. Leibowitch.** 2003. Longitudinal monitoring of 2-long terminal repeat circles in peripheral blood mononuclear cells from patients with chronic HIV-1 infection. Aids **17:**645-652.

24. **Burniston, M. T., A. Cimarelli, J. Colgan, S. P. Curtis, and J. Luban.** 1999. Human immunodeficiency virus type 1 Gag polyprotein multimerization requires the nucleocapsid domain and RNA and is promoted by the capsid-dimer interface and the basic region of matrix protein. J Virol **73:**8527-8540.

25. **Butler, S. L., E. P. Johnson, and F. D. Bushman.** 2002. Human immunodeficiency virus cDNA metabolism: notable stability of two-long terminal repeat circles. J Virol **76:**3739-3747.

26. **Butsch, M., and K. Boris-Lawrie.** 2002. Destiny of unspliced retroviral RNA: ribosome and/or virion? J Virol **76:**3089-3094.

27. **Campbell, S., and A. Rein.** 1999. In vitro assembly properties of human immunodeficiency virus type 1 Gag protein lacking the p6 domain. J Virol **73:**2270-2279.

28. **Campbell, S., and V. M. Vogt.** 1995. Self-assembly in vitro of purified CA-NC proteins from Rous sarcoma virus and human immunodeficiency virus type 1. J Virol **69:**6487-6497.

29. **Cartier, N., S. Hacein-Bey-Abina, C. C. Bartholomae, G. Veres, M. Schmidt, I. Kutschera, M. Vidaud, U. Abel, L. Dal-Cortivo, L. Caccavelli, N. Mahlaoui, V. Kiermer, D. Mittelstaedt, C. Bellesme, N. Lahlou, F. Lefrere, S. Blanche, M. Audit, E. Payen, P. Leboulch, B. l'Homme, P. Bougneres, C. Von Kalle, A.**

Fischer, M. Cavazzana-Calvo, and P. Aubourg. 2009. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science **326:**818-823.

30. **Cavazzana-Calvo, M., E. Payen, O. Negre, G. Wang, K. Hehir, F. Fusil, J. Down, M. Denaro, T. Brady, K. Westerman, R. Cavallesco, B. Gillet-Legrand, L. Caccavelli, R. Sgarra, L. Maouche-Chretien, F. Bernaudin, R. Girot, R. Dorazio, G. J. Mulder, A. Polack, A. Bank, J. Soulier, J. Larghero, N. Kabbara, B. Dalle, B. Gourmel, G. Socie, S. Chretien, N. Cartier, P. Aubourg, A. Fischer, K. Cornetta, F. Galacteros, Y. Beuzard, E. Gluckman, F. Bushman, S. Hacein-Bey-Abina, and P. Leboulch.** 2010. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature **467:**318-322.

31. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of splice sites. Cell **59:**789-795.

32. **Chang, K. W., J. G. Julias, W. G. Alvord, J. Oh, and S. H. Hughes.** 2005. Alternate polypurine tracts (PPTs) affect the rous sarcoma virus RNase H cleavage specificity and reveal a preferential cleavage following a GA dinucleotide sequence at the PPT-U3 junction. J Virol **79:**13694-13704.

33. **Chang, K. W., J. Oh, W. G. Alvord, and S. H. Hughes.** 2008. The effects of alternate polypurine tracts (PPTs) and mutations of sequences adjacent to the PPT on viral replication and cleavage specificity of the Rous sarcoma virus reverse transcriptase. J Virol **82:**8592-8604.

34. **Chun, T. W., L. Carruth, D. Finzi, X. Shen, J. A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T. C. Quinn, Y. H. Kuo, R.**

Brookmeyer, M. A. Zeiger, P. Barditch-Crovo, and R. F. Siliciano. 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature **387:**183-188.

35. **Cimarelli, A., S. Sandin, S. Hoglund, and J. Luban.** 2000. Basic residues in human immunodeficiency virus type 1 nucleocapsid promote virion assembly via interaction with RNA. J Virol **74:**3046-3057.

36. **Clavel, F., and J. M. Orenstein.** 1990. A mutant of human immunodeficiency virus with reduced RNA packaging and abnormal particle morphology. J Virol **64:**5230-5234.

37. **Clever, J. L., D. A. Eckstein, and T. G. Parslow.** 1999. Genetic dissociation of the encapsidation and reverse transcription functions in the 5' R region of human immunodeficiency virus type 1. J Virol **73:**101-109.

38. **Clever, J. L., D. Miranda, Jr., and T. G. Parslow.** 2002. RNA structure and packaging signals in the 5' leader region of the human immunodeficiency virus type 1 genome. J Virol **76:**12381-12387.

39. **Cochrane, A. W., C. H. Chen, and C. A. Rosen.** 1990. Specific interaction of the human immunodeficiency virus Rev protein with a structured region in the env mRNA. Proc Natl Acad Sci U S A **87:**1198-1202.

40. **Cochrane, A. W., K. S. Jones, S. Beidas, P. J. Dillon, A. M. Skalka, and C. A. Rosen.** 1991. Identification and characterization of intragenic sequences which repress human immunodeficiency virus structural gene expression. J Virol **65:**5305-5313.

41. **Cochrane, A. W., M. T. McNally, and A. J. Mouland.** 2006. The retrovirus RNA trafficking granule: from birth to maturity. Retrovirology **3:**18.

42. **Cockrell, A. S., and T. Kafri.** 2007. Gene delivery by lentivirus vectors. Mol Biotechnol **36:**184-204.

43. **Cockrell, A. S., and T. Kafri.** 2003. HIV-1 vectors: fulfillment of expectations, further advancements, and still a way to go. Curr HIV Res **1:**419-439.

44. **Cockrell, A. S., H. Ma, K. Fu, T. J. McCown, and T. Kafri.** 2006. A trans-lentiviral packaging cell line for high-titer conditional self-inactivating HIV-1 vectors. Mol Ther **14:**276-284.

45. **Cockrell, A. S., H. van Praag, N. Santistevan, H. Ma, and T. Kafri.** 2011. The HIV-1 Rev/RRE system is required for HIV-1 5' UTR cis elements to augment encapsidation of heterologous RNA into HIV-1 viral particles. Retrovirology **8:**51.

46. **Coffin, J. M., S. M. Hughes, and H. E. Varmus.** 1997. Retroviruses.

47. **Cullen, B. R.** 2003. Nuclear mRNA export: insights from virology. Trends Biochem Sci **28:**419-424.

48. **D'Agostino, D. M., B. K. Felber, J. E. Harrison, and G. N. Pavlakis.** 1992. The Rev protein of human immunodeficiency virus type 1 promotes polysomal association and translation of gag/pol and vpu/env mRNAs. Mol Cell Biol **12:**1375-1386.

49. **D'Souza, V., and M. F. Summers.** 2005. How retroviruses select their genomes. Nat Rev Microbiol **3:**643-655.

50. **Damgaard, C. K., E. S. Andersen, B. Knudsen, J. Gorodkin, and J. Kjems.** 2004. RNA interactions in the 5' region of the HIV-1 genome. J Mol Biol **336:**369-379.

51. **Das, A. T., B. Klaver, and B. Berkhout.** 1995. Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural tRNA(3Lys). J Virol **69:**3090-3097.

52. **DiMattia, M. A., N. R. Watts, S. J. Stahl, C. Rader, P. T. Wingfield, D. I. Stuart, A. C. Steven, and J. M. Grimes.** 2010. Implications of the HIV-1 Rev dimer structure at 3.2 A resolution for multimeric binding to the Rev response element. Proc Natl Acad Sci U S A **107:**5810-5814.

53. **Dorman, N., and A. Lever.** 2000. Comparison of viral genomic RNA sorting mechanisms in human immunodeficiency virus type 1 (HIV-1), HIV-2, and Moloney murine leukemia virus. J Virol **74:**11413-11417.

54. **Duffy, S., L. A. Shackelton, and E. C. Holmes.** 2008. Rates of evolutionary change in viruses: patterns and determinants. Nat Rev Genet **9:**267-276.

55. **Emerman, M., R. Vazeux, and K. Peden.** 1989. The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. Cell **57:**1155-1165.

56. **Fang, J., E. Acheampong, R. Dave, F. Wang, M. Mukhtar, and R. J. Pomerantz.** 2005. The RNA helicase DDX1 is involved in restricted HIV-1 Rev function in human astrocytes. Virology **336:**299-307.

57. **Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46:**807-817.

58. **Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj.** 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell **90:**1051-1060.

59. **Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and E. Nishida.** 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature **390:**308-311.

60. **Fuller, M., and D. S. Anson.** 2001. Helper plasmids for production of HIV-1-derived vectors. Hum Gene Ther **12:**2081-2093.

61. **Furfine, E. S., and J. E. Reardon.** 1991. Human immunodeficiency virus reverse transcriptase ribonuclease H: specificity of tRNA(Lys3)-primer excision. Biochemistry **30:**7041-7046.

62. **Gallego, J., J. Greatorex, H. Zhang, B. Yang, S. Arunachalam, J. Fang, J. Seamons, S. Lea, R. J. Pomerantz, and A. M. Lever.** 2003. Rev binds specifically to a purine loop in the SL1 region of the HIV-1 leader RNA. J Biol Chem **278:**40385-40391.

63. **Garzino-Demo, A., R. C. Gallo, and S. K. Arya.** 1995. Human immunodeficiency virus type 2 (HIV-2): packaging signal and associated negative regulatory element. Hum Gene Ther **6:**177-184.

64. **Gherghe, C., C. W. Leonard, R. J. Gorelick, and K. M. Weeks.** 2010. Secondary structure of the mature ex virio Moloney murine leukemia virus genomic RNA dimerization domain. J Virol **84:**898-906.

65. **Gherghe, C., T. Lombo, C. W. Leonard, S. A. Datta, J. W. Bess, Jr., R. J. Gorelick, A. Rein, and K. M. Weeks.** 2010. Definition of a high-affinity Gag recognition structure mediating packaging of a retroviral RNA genome. Proc Natl Acad Sci U S A **107:**19248-19253.

66. **Graf, M., A. Bojak, L. Deml, K. Bieler, H. Wolf, and R. Wagner.** 2000. Concerted action of multiple cis-acting sequences is required for Rev dependence of late human immunodeficiency virus type 1 gene expression. J Virol **74:**10822-10826.

67. **Greatorex, J. S., E. A. Palmer, R. J. Pomerantz, J. A. Dangerfield, and A. M. Lever.** 2006. Mutation of the Rev-binding loop in the human immunodeficiency virus 1 leader causes a replication defect characterized by altered RNA trafficking and packaging. J Gen Virol **87:**3039-3044.

68. **Grewe, B., and K. Uberla.** 2010. The human immunodeficiency virus type 1 Rev protein: menage a trois during the early phase of the lentiviral replication cycle. J Gen Virol **91:**1893-1897.

69. **Griffin, S. D., J. F. Allen, and A. M. Lever.** 2001. The major human immunodeficiency virus type 2 (HIV-2) packaging signal is present on all HIV-2 RNA species: cotranslational RNA encapsidation and limitation of Gag protein confer specificity. J Virol **75:**12058-12069.

70. **Groom, H. C., E. C. Anderson, J. A. Dangerfield, and A. M. Lever.** 2009. Rev regulates translation of human immunodeficiency virus type 1 RNAs. J Gen Virol **90:**1141-1147.

71. **Groom, H. C., E. C. Anderson, and A. M. Lever.** 2009. Rev: beyond nuclear export. J Gen Virol **90:**1303-1318.

72. **Gross, I., H. Hohenberg, and H. G. Krausslich.** 1997. In vitro assembly properties of purified bacterially expressed capsid proteins of human immunodeficiency virus. Eur J Biochem **249:**592-600.

73. **Gruter, P., C. Tabernero, C. von Kobbe, C. Schmitt, C. Saavedra, A. Bachi, M. Wilm, B. K. Felber, and E. Izaurralde.** 1998. TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. Mol Cell **1:**649-659.

74. **Guzik, B. W., L. Levesque, S. Prasad, Y. C. Bor, B. E. Black, B. M. Paschal, D. Rekosh, and M. L. Hammarskjold.** 2001. NXT1 (p15) is a crucial cellular cofactor in TAP-dependent export of intron-containing RNA in mammalian cells. Mol Cell Biol **21:**2545-2554.

75. **Hacein-Bey-Abina, S., C. von Kalle, M. Schmidt, F. Le Deist, N. Wulffraat, E. McIntyre, I. Radford, J. L. Villeval, C. C. Fraser, M. Cavazzana-Calvo, and A. Fischer.** 2003. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med **348:**255-256.

76. **Hacein-Bey-Abina, S., C. Von Kalle, M. Schmidt, M. P. McCormack, N. Wulffraat, P. Leboulch, A. Lim, C. S. Osborne, R. Pawliuk, E. Morillon, R. Sorensen, A. Forster, P. Fraser, J. I. Cohen, G. de Saint Basile, I. Alexander, U. Wintergerst, T. Frebourg, A. Aurias, D. Stoppa-Lyonnet, S. Romana, I. Radford-Weiss, F. Gross, F. Valensi, E. Delabesse, E. Macintyre, F. Sigaux, J. Soulier, L. E. Leiva, M. Wissler, C. Prinz, T. H. Rabbitts, F. Le Deist, A. Fischer, and M. Cavazzana-Calvo.** 2003. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science **302:**415-419.

77. **Hadzopoulou-Cladaras, M., B. K. Felber, C. Cladaras, A. Athanassopoulos, A. Tse, and G. N. Pavlakis.** 1989. The rev (trs/art) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a cis-acting sequence in the env region. J Virol **63:**1265-1274.

78. **Hajjar, A. M., and M. L. Linial.** 1993. A model system for nonhomologous recombination between retroviral and cellular RNA. J Virol **67:**3845-3853.

79. **Harrison, G. P., G. Miele, E. Hunter, and A. M. Lever.** 1998. Functional analysis of the core human immunodeficiency virus type 1 packaging signal in a permissive cell line. J Virol **72:**5886-5896.

80. **He, J. J., J. Henao-Mejia, and Y. Liu.** 2009. Sam68 functions in nuclear export and translation of HIV-1 RNA. RNA Biol **6:**384-386.

81. **Helga-Maria, C., M. L. Hammarskjold, and D. Rekosh.** 1999. An intact TAR element and cytoplasmic localization are necessary for efficient packaging of human immunodeficiency virus type 1 genomic RNA. J Virol **73:**4127-4135.

82. **Henderson, B. R., and P. Percipalle.** 1997. Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta. J Mol Biol **274:**693-707.

83. **Hibbert, C. S., J. Mirro, and A. Rein.** 2004. mRNA molecules containing murine leukemia virus packaging signals are encapsidated as dimers. J Virol **78:**10927-10938.

84. **Hill, M. K., M. Shehu-Xhilaga, S. M. Campbell, P. Poumbourios, S. M. Crowe, and J. Mak.** 2003. The dimer initiation sequence stem-loop of human immunodeficiency virus type 1 is dispensable for viral replication in peripheral blood mononuclear cells. J Virol **77:**8329-8335.

85. **Holmes, E. C.** 2011. What does virus evolution tell us about virus origins? J Virol **85:**5247-5251.

86. **Hope, T. J., B. L. Bond, D. McDonald, N. P. Klein, and T. G. Parslow.** 1991. Effector domains of human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex are functionally interchangeable and share an essential peptide motif. J Virol **65:**6001-6007.

87. **Hope, T. J., X. J. Huang, D. McDonald, and T. G. Parslow.** 1990. Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping cryptic functions of the arginine-rich motif. Proc Natl Acad Sci U S A **87:**7787-7791.

88. **Hope, T. J., D. McDonald, X. J. Huang, J. Low, and T. G. Parslow.** 1990. Mutational analysis of the human immunodeficiency virus type 1 Rev transactivator: essential residues near the amino terminus. J Virol **64:**5360-5366.

89. **Houzet, L., J. C. Paillart, F. Smagulova, S. Maurel, Z. Morichaud, R. Marquet, and M. Mougel.** 2007. HIV controls the selective packaging of genomic, spliced viral and cellular RNAs into virions through different mechanisms. Nucleic Acids Res **35:**2695-2704.

90. **Hutchinson, E. C., J. C. von Kirchbach, J. R. Gog, and P. Digard.** 2010. Genome packaging in influenza A virus. J Gen Virol **91:**313-328.

91. **Hutten, S., and R. H. Kehlenbach.** 2007. CRM1-mediated nuclear export: to the pore and beyond. Trends Cell Biol **17:**193-201.

92. **Ikeda, Y., Y. Takeuchi, F. Martin, F. L. Cosset, K. Mitrophanous, and M. Collins.** 2003. Continuous high-titer HIV-1 vector production. Nat Biotechnol **21:**569-572.

93. **Jin, J., T. Sturgeon, C. Chen, S. C. Watkins, O. A. Weisz, and R. C. Montelaro.** 2007. Distinct intracellular trafficking of equine infectious anemia virus and human

immunodeficiency virus type 1 Gag during viral assembly and budding revealed by bimolecular fluorescence complementation assays. J Virol **81:**11226-11235.

94. **Jin, J., T. Sturgeon, O. A. Weisz, W. Mothes, and R. C. Montelaro.** 2009. HIV-1 matrix dependent membrane targeting is regulated by Gag mRNA trafficking. PLoS One **4:**e6551.

95. **Johnson, S. F., and A. Telesnitsky.** 2010. Retroviral RNA dimerization and packaging: the what, how, when, where, and why. PLoS Pathog **6:**e1001007.

96. **Jouvenet, N., P. D. Bieniasz, and S. M. Simon.** 2008. Imaging the biogenesis of individual HIV-1 virions in live cells. Nature **454:**236-240.

97. **Jouvenet, N., S. M. Simon, and P. D. Bieniasz.** 2009. Imaging the interaction of HIV-1 genomes and Gag during assembly of individual viral particles. Proc Natl Acad Sci U S A **106:**19114-19119.

98. **Kammler, S., C. Leurs, M. Freund, J. Krummheuer, K. Seidel, T. O. Tange, M. K. Lund, J. Kjems, A. Scheid, and H. Schaal.** 2001. The sequence complementarity between HIV-1 5' splice site SD4 and U1 snRNA determines the steady-state level of an unstable env pre-mRNA. Rna **7:**421-434.

99. **Kantor, B., M. Bayer, H. Ma, J. Samulski, C. Li, T. McCown, and T. Kafri.** 2011. Notable Reduction in Illegitimate Integration Mediated by a PPT-deleted, Nonintegrating Lentiviral Vector. Mol Ther **19:**547-556.

100. **Kantor, B., H. Ma, J. Webster-Cyriaque, P. E. Monahan, and T. Kafri.** 2009. Epigenetic activation of unintegrated HIV-1 genomes by gut-associated short chain fatty acids and its implications for HIV infection. Proc Natl Acad Sci U S A **106:**18786-18791.

101. **Kaye, J. F., and A. M. Lever.** 1999. Human immunodeficiency virus types 1 and 2 differ in the predominant mechanism used for selection of genomic RNA for encapsidation. J Virol **73:**3023-3031.

102. **Kaye, J. F., J. H. Richardson, and A. M. Lever.** 1995. cis-acting sequences involved in human immunodeficiency virus type 1 RNA packaging. J Virol **69:**6588-6592.

103. **Kemler, I., I. Azmi, and E. M. Poeschla.** 2004. The critical role of proximal gag sequences in feline immunodeficiency virus genome encapsidation. Virology **327:**111-120.

104. **Kenyon, J. C., S. J. Tanner, M. Legiewicz, P. S. Phillip, T. A. Rizvi, S. F. Le Grice, and A. M. Lever.** 2011. SHAPE analysis of the FIV Leader RNA reveals a structural switch potentially controlling viral packaging and genome dimerization. Nucleic Acids Res **39:**6692-6704.

105. **Keryer-Bibens, C., C. Barreau, and H. B. Osborne.** 2008. Tethering of proteins to RNAs by bacteriophage proteins. Biol Cell **100:**125-138.

106. **King, J. A., J. M. Bridger, F. Gounari, P. Lichter, T. F. Schulz, V. Schirrmacher, and K. Khazaie.** 1998. The extended packaging sequence of MoMLV contains a constitutive mRNA nuclear export function. FEBS Lett **434:**367-371.

107. **Kjems, J., A. D. Frankel, and P. A. Sharp.** 1991. Specific regulation of mRNA splicing in vitro by a peptide from HIV-1 Rev. Cell **67:**169-178.

108. **Kjems, J., and P. A. Sharp.** 1993. The basic domain of Rev from human immunodeficiency virus type 1 specifically blocks the entry of U4/U6.U5 small nuclear ribonucleoprotein in spliceosome assembly. J Virol **67:**4769-4776.

109. **Kol, N., M. Gladnikoff, D. Barlam, R. Z. Shneck, A. Rein, and I. Rousso.** 2006. Mechanical properties of murine leukemia virus particles: effect of maturation. Biophys J **91:**767-774.

110. **Kol, N., Y. Shi, M. Tsvitov, D. Barlam, R. Z. Shneck, M. S. Kay, and I. Rousso.** 2007. A stiffness switch in human immunodeficiency virus. Biophys J **92:**1777-1783.

111. **Kotsopoulou, E., V. N. Kim, A. J. Kingsman, S. M. Kingsman, and K. A. Mitrophanous.** 2000. A Rev-independent human immunodeficiency virus type 1 (HIV-1)-based vector that exploits a codon-optimized HIV-1 gag-pol gene. J Virol **74:**4839-4852.

112. **Krupovic, M., and D. H. Bamford.** 2010. Order to the viral universe. J Virol **84:**12476-12479.

113. **Krupovic, M., and D. H. Bamford.** 2008. Virus evolution: how far does the double beta-barrel viral lineage extend? Nat Rev Microbiol **6:**941-948.

114. **Krupovic, M., P. Forterre, and D. H. Bamford.** 2010. Comparative analysis of the mosaic genomes of tailed archaeal viruses and proviruses suggests common themes for virion architecture and assembly with tailed viruses of bacteria. J Mol Biol **397:**144-160.

115. **Kula, A., J. Guerra, A. Knezevich, D. Kleva, M. P. Myers, and A. Marcello.** 2011. Characterization of the HIV-1 RNA associated proteome identifies Matrin 3 as a nuclear cofactor of Rev function. Retrovirology **8:**60.

116. **Kutluay, S. B., and P. D. Bieniasz.** 2010. Analysis of the initiating events in HIV-1 particle assembly and genome packaging. PLoS Pathog **6:**e1001200.

117. **Laham-Karam, N., and E. Bacharach.** 2007. Transduction of human immunodeficiency virus type 1 vectors lacking encapsidation and dimerization signals. J Virol **81:**10687-10698.

118. **Lanchy, J. M., J. D. Ivanovitch, and J. S. Lodmell.** 2003. A structural linkage between the dimerization and encapsidation signals in HIV-2 leader RNA. Rna **9:**1007-1018.

119. **Lesnik, E. A., R. Sampath, and D. J. Ecker.** 2002. Rev response elements (RRE) in lentiviruses: an RNAMotif algorithm-based strategy for RRE prediction. Med Res Rev **22:**617-636.

120. **Lever, A., H. Gottlinger, W. Haseltine, and J. Sodroski.** 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. J Virol **63:**4085-4087.

121. **Lever, A. M.** 2007. HIV-1 RNA packaging. Adv Pharmacol **55:**1-32.

122. **Levin, J. G., and M. J. Rosenak.** 1976. Synthesis of murine leukemia virus proteins associated with virions assembled in actinomycin D-treated cells: evidence for persistence of viral messenger RNA. Proc Natl Acad Sci U S A **73:**1154-1158.

123. **Linial, M.** 1987. Creation of a processed pseudogene by retroviral infection. Cell **49:**93-102.

124. **Lu, X. B., J. Heimer, D. Rekosh, and M. L. Hammarskjold.** 1990. U1 small nuclear RNA plays a direct role in the formation of a rev-regulated human immunodeficiency virus env mRNA that remains unspliced. Proc Natl Acad Sci U S A **87:**7598-7602.

125. **Lucke, S., T. Grunwald, and K. Uberla.** 2005. Reduced mobilization of Rev-responsive element-deficient lentiviral vectors. J Virol **79:**9359-9362.

126. **Ma, J., L. Rong, Y. Zhou, B. B. Roy, J. Lu, L. Abrahamyan, A. J. Mouland, Q. Pan, and C. Liang.** 2008. The requirement of the DEAD-box protein DDX24 for the packaging of human immunodeficiency virus type 1 RNA. Virology **375:**253-264.

127. **Ma, Y. M., and V. M. Vogt.** 2004. Nucleic acid binding-induced Gag dimerization in the assembly of Rous sarcoma virus particles in vitro. J Virol **78:**52-60.

128. **Ma, Y. M., and V. M. Vogt.** 2002. Rous sarcoma virus Gag protein-oligonucleotide interaction suggests a critical role for protein dimer formation in assembly. J Virol **76:**5452-5462.

129. **Maldarelli, F., M. A. Martin, and K. Strebel.** 1991. Identification of posttranscriptionally active inhibitory sequences in human immunodeficiency virus type 1 RNA: novel level of gene regulation. J Virol **65:**5732-5743.

130. **Malim, M. H., S. Bohnlein, R. Fenrick, S. Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. Functional comparison of the Rev trans-activators encoded by different primate immunodeficiency virus species. Proc Natl Acad Sci U S A **86:**8222-8226.

131. **Malim, M. H., S. Bohnlein, J. Hauber, and B. R. Cullen.** 1989. Functional dissection of the HIV-1 Rev trans-activator--derivation of a trans-dominant repressor of Rev function. Cell **58:**205-214.

132. **Malim, M. H., J. Hauber, S. Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature **338:**254-257.

133. **Malim, M. H., D. F. McCarn, L. S. Tiley, and B. R. Cullen.** 1991. Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. J Virol **65:**4248-4254.

134. **Mann, R., and D. Baltimore.** 1985. Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNAs. J Virol **54:**401-407.

135. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33:**153-159.

136. **Masuda, T., M. J. Kuroda, and S. Harada.** 1998. Specific and independent recognition of U3 and U5 att sites by human immunodeficiency virus type 1 integrase in vivo. J Virol **72:**8396-8402.

137. **Matrai, J., A. Cantore, C. C. Bartholomae, A. Annoni, W. Wang, A. Acosta-Sanchez, E. Samara-Kuko, L. De Waele, L. Ma, P. Genovese, M. Damo, A. Arens, K. Goudy, T. C. Nichols, C. von Kalle, L. C. MK, M. G. Roncarolo, M. Schmidt, T. Vandendriessche, and L. Naldini.** 2011. Hepatocyte-targeted expression by integrase-defective lentiviral vectors induces antigen-specific tolerance in mice with low genotoxic risk. Hepatology **53:**1696-1707.

138. **McBride, M. S., and A. T. Panganiban.** 1996. The human immunodeficiency virus type 1 encapsidation site is a multipartite RNA element composed of functional hairpin structures. J Virol **70:**2963-2973.

139. **McBride, M. S., M. D. Schwartz, and A. T. Panganiban.** 1997. Efficient encapsidation of human immunodeficiency virus type 1 vectors and further characterization of cis elements required for encapsidation. J Virol **71:**4544-4554.

140. **McCann, E. M., and A. M. Lever.** 1997. Location of cis-acting signals important for RNA encapsidation in the leader sequence of human immunodeficiency virus type 2. J Virol **71:**4133-4137.

141. **McCown, T. J.** 2006. Adeno-associated virus-mediated expression and constitutive secretion of galanin suppresses limbic seizure activity in vivo. Mol Ther **14:**63-68.

142. **McDonald, D., T. J. Hope, and T. G. Parslow.** 1992. Posttranscriptional regulation by the human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex proteins through a heterologous RNA binding site. J Virol **66:**7232-7238.

143. **McDonald, S. M., and J. T. Patton.** 2011. Assortment and packaging of the segmented rotavirus genome. Trends Microbiol **19:**136-144.

144. **Mettenleiter, T. C., B. G. Klupp, and H. Granzow.** 2009. Herpesvirus assembly: an update. Virus Res **143:**222-234.

145. **Meyer, B. E., J. L. Meinkoth, and M. H. Malim.** 1996. Nuclear transport of human immunodeficiency virus type 1, visna virus, and equine infectious anemia virus Rev proteins: identification of a family of transferable nuclear export signals. J Virol **70:**2350-2359.

146. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. Biotechniques **7:**980-982, 984-986, 989-990.

147. **Modem, S., and T. R. Reddy.** 2008. An anti-apoptotic protein, Hax-1, inhibits the HIV-1 rev function by altering its sub-cellular localization. J Cell Physiol **214:**14-19.

148. **Moore, M. D., and W. S. Hu.** 2009. HIV-1 RNA dimerization: It takes two to tango. AIDS Rev **11:**91-102.

149. **Muriaux, D., and J. L. Darlix.** 2010. Properties and functions of the nucleocapsid protein in virus assembly. RNA Biol **7**.

150. **Muriaux, D., J. Mirro, D. Harvin, and A. Rein.** 2001. RNA is a structural element in retrovirus particles. Proc Natl Acad Sci U S A **98:**5246-5251.

151. **Muriaux, D., J. Mirro, K. Nagashima, D. Harvin, and A. Rein.** 2002. Murine leukemia virus nucleocapsid mutant particles lacking viral RNA encapsidate ribosomes. J Virol **76:**11405-11413.

152. **Muriaux, D., and A. Rein.** 2003. Encapsidation and transduction of cellular genes by retroviruses. Front Biosci **8:**d135-142.

153. **Nasioulas, G., A. S. Zolotukhin, C. Tabernero, L. Solomin, C. P. Cunningham, G. N. Pavlakis, and B. K. Felber.** 1994. Elements distinct from human immunodeficiency virus type 1 splice sites are responsible for the Rev dependence of env mRNA. J Virol **68:**2986-2993.

154. **Naviaux, R. K., E. Costanzi, M. Haas, and I. M. Verma.** 1996. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. J Virol **70:**5701-5705.

155. **Neville, M., F. Stutz, L. Lee, L. I. Davis, and M. Rosbash.** 1997. The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. Curr Biol **7:**767-775.

156. **Ni, N., O. A. Nikolaitchik, K. A. Dilley, J. Chen, A. Galli, W. Fu, V. V. Prasad, R. G. Ptak, V. K. Pathak, and W. S. Hu.** 2011. Mechanisms of human

immunodeficiency virus type 2 RNA packaging: efficient trans packaging and selection of RNA copackaging partners. J Virol **85:**7603-7612.

157. **Nienhuis, A. W., C. E. Dunbar, and B. P. Sorrentino.** 2006. Genotoxicity of retroviral integration in hematopoietic cells. Mol Ther **13:**1031-1049.

158. **Nightingale, S. J., R. P. Hollis, K. A. Pepper, D. Petersen, X. J. Yu, C. Yang, I. Bahner, and D. B. Kohn.** 2006. Transient gene expression by nonintegrating lentiviral vectors. Mol Ther **13:**1121-1132.

159. **Oh, J., M. J. McWilliams, J. G. Julias, and S. H. Hughes.** 2008. Mutations in the U5 region adjacent to the primer binding site affect tRNA cleavage by human immunodeficiency virus type 1 reverse transcriptase in vivo. J Virol **82:**719-727.

160. **Olsen, H. S., P. Nelbock, A. W. Cochrane, and C. A. Rosen.** 1990. Secondary structure is the major determinant for interaction of HIV rev protein with RNA. Science **247:**845-848.

161. **Onafuwa-Nuga, A. A., S. R. King, and A. Telesnitsky.** 2005. Nonrandom packaging of host RNAs in moloney murine leukemia virus. J Virol **79:**13528-13537.

162. **Onafuwa-Nuga, A. A., A. Telesnitsky, and S. R. King.** 2006. 7SL RNA, but not the 54-kd signal recognition particle protein, is an abundant component of both infectious HIV-1 and minimal virus-like particles. Rna **12:**542-546.

163. **Ossareh-Nazari, B., F. Bachelerie, and C. Dargemont.** 1997. Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science **278:**141-144.

164. **Otero, G. C., M. E. Harris, J. E. Donello, and T. J. Hope.** 1998. Leptomycin B inhibits equine infectious anemia virus Rev and feline immunodeficiency virus rev

function but not the function of the hepatitis B virus posttranscriptional regulatory element. J Virol **72:**7593-7597.

165. **Ott, D. E., L. V. Coren, E. N. Chertova, T. D. Gagliardi, K. Nagashima, R. C. Sowder, 2nd, D. T. Poon, and R. J. Gorelick.** 2003. Elimination of protease activity restores efficient virion production to a human immunodeficiency virus type 1 nucleocapsid deletion mutant. J Virol **77:**5547-5556.

166. **Ott, D. E., L. V. Coren, and T. D. Gagliardi.** 2005. Redundant roles for nucleocapsid and matrix RNA-binding sequences in human immunodeficiency virus type 1 assembly. J Virol **79:**13839-13847.

167. **Paillart, J. C., M. Dettenhofer, X. F. Yu, C. Ehresmann, B. Ehresmann, and R. Marquet.** 2004. First snapshots of the HIV-1 RNA structure in infected cells and in virions. J Biol Chem **279:**48397-48403.

168. **Paillart, J. C., M. Shehu-Xhilaga, R. Marquet, and J. Mak.** 2004. Dimerization of retroviral RNA genomes: an inseparable pair. Nat Rev Microbiol **2:**461-472.

169. **Pang, S., Y. Koyanagi, S. Miles, C. Wiley, H. V. Vinters, and I. S. Chen.** 1990. High levels of unintegrated HIV-1 DNA in brain tissue of AIDS dementia patients. Nature **343:**85-89.

170. **Patel, J., S. W. Wang, E. Izmailova, and A. Aldovini.** 2003. The simian immunodeficiency virus 5' untranslated leader sequence plays a role in intracellular viral protein accumulation and in RNA packaging. J Virol **77:**6284-6292.

171. **Paxinos, G., and C. Watson.** 1986. The rat brain in stereotaxic coordinates.

172. **Perales, C., L. Carrasco, and M. E. Gonzalez.** 2005. Regulation of HIV-1 env mRNA translation by Rev protein. Biochim Biophys Acta **1743:**169-175.

173. **Philippe, S., C. Sarkis, M. Barkats, H. Mammeri, C. Ladroue, C. Petit, J. Mallet, and C. Serguera.** 2006. Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. Proc Natl Acad Sci U S A **103:**17684-17689.

174. **Phillips, T. R., C. Lamont, D. A. Konings, B. L. Shacklett, C. A. Hamson, P. A. Luciw, and J. H. Elder.** 1992. Identification of the Rev transactivation and Rev-responsive elements of feline immunodeficiency virus. J Virol **66:**5464-5471.

175. **Pierson, T. C., T. L. Kieffer, C. T. Ruff, C. Buck, S. J. Gange, and R. F. Siliciano.** 2002. Intrinsic stability of episomal circles formed during human immunodeficiency virus type 1 replication. J Virol **76:**4138-4144.

176. **Pollard, V. W., and M. H. Malim.** 1998. The HIV-1 Rev protein. Annu Rev Microbiol **52:**491-532.

177. **Pond, S. J., W. K. Ridgeway, R. Robertson, J. Wang, and D. P. Millar.** 2009. HIV-1 Rev protein assembles on viral RNA one molecule at a time. Proc Natl Acad Sci U S A **106:**1404-1408.

178. **Poole, E., P. Strappe, H. P. Mok, R. Hicks, and A. M. Lever.** 2005. HIV-1 Gag-RNA interaction occurs at a perinuclear/centrosomal site; analysis by confocal microscopy and FRET. Traffic **6:**741-755.

179. **Rahim, A. A., A. M. Wong, S. J. Howe, S. M. Buckley, A. D. Acosta-Saltos, K. E. Elston, N. J. Ward, N. J. Philpott, J. D. Cooper, P. N. Anderson, S. N. Waddington, A. J. Thrasher, and G. Raivich.** 2009. Efficient gene delivery to the adult and fetal CNS using pseudotyped non-integrating lentiviral vectors. Gene Ther **16:**509-520.

180. **Reddy, T. R., W. Xu, J. K. Mau, C. D. Goodwin, M. Suhasini, H. Tang, K. Frimpong, D. W. Rose, and F. Wong-Staal.** 1999. Inhibition of HIV replication by dominant negative mutants of Sam68, a functional homolog of HIV-1 Rev. Nat Med **5:**635-642.

181. **Richardson, J. H., L. A. Child, and A. M. Lever.** 1993. Packaging of human immunodeficiency virus type 1 RNA requires cis-acting sequences outside the 5' leader region. J Virol **67:**3997-4005.

182. **Riviere, L., J. L. Darlix, and A. Cimarelli.** 2010. Analysis of the viral elements required in the nuclear import of HIV-1 DNA. J Virol **84:**729-739.

183. **Rizvi, T. A., J. C. Kenyon, J. Ali, S. J. Aktar, P. S. Phillip, A. Ghazawi, F. Mustafa, and A. M. Lever.** 2010. Optimal packaging of FIV genomic RNA depends upon a conserved long-range interaction and a palindromic sequence within gag. J Mol Biol **403:**103-119.

184. **Rizvi, T. A., and A. T. Panganiban.** 1993. Simian immunodeficiency virus RNA is efficiently encapsidated by human immunodeficiency virus type 1 particles. J Virol **67:**2681-2688.

185. **Roitman-Shemer, V., J. Stokrova, J. Forstova, and A. Oppenheim.** 2007. Assemblages of simian virus 40 capsid proteins and viral DNA visualized by electron microscopy. Biochem Biophys Res Commun **353:**424-430.

186. **Roos, W. H., I. L. Ivanovska, A. Evilevitch, and G. J. Wuite.** 2007. Viral capsids: mechanical characteristics, genome packaging and delivery mechanisms. Cell Mol Life Sci **64:**1484-1497.

187. **Rosen, C. A., E. Terwilliger, A. Dayton, J. G. Sodroski, and W. A. Haseltine.** 1988. Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus. Proc Natl Acad Sci U S A **85:**2071-2075.

188. **Rulli, S. J., Jr., C. S. Hibbert, J. Mirro, T. Pederson, S. Biswal, and A. Rein.** 2007. Selective and nonselective packaging of cellular RNAs in retrovirus particles. J Virol **81:**6623-6631.

189. **Sanchez-Velar, N., E. B. Udofia, Z. Yu, and M. L. Zapp.** 2004. hRIP, a cellular cofactor for Rev function, promotes release of HIV RNAs from the perinuclear region. Genes Dev **18:**23-34.

190. **Schwartz, S., M. Campbell, G. Nasioulas, J. Harrison, B. K. Felber, and G. N. Pavlakis.** 1992. Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent gag expression. J Virol **66:**7176-7182.

191. **Sherer, N. M., C. M. Swanson, S. Papaioannou, and M. H. Malim.** 2009. Matrix mediates the functional link between human immunodeficiency virus type 1 RNA nuclear export elements and the assembly competency of Gag in murine cells. J Virol **83:**8525-8535.

192. **Sinck, L., D. Richer, J. Howard, M. Alexander, D. F. Purcell, R. Marquet, and J. C. Paillart.** 2007. In vitro dimerization of human immunodeficiency virus type 1 (HIV-1) spliced RNAs. Rna **13:**2141-2150.

193. **Smagulova, F., S. Maurel, Z. Morichaud, C. Devaux, M. Mougel, and L. Houzet.** 2005. The highly structured encapsidation signal of MuLV RNA is involved in the nuclear export of its unspliced RNA. J Mol Biol **354:**1118-1128.

194. **Smith, J. S., and M. J. Roth.** 1992. Specificity of human immunodeficiency virus-1 reverse transcriptase-associated ribonuclease H in removal of the minus-strand primer, tRNA(Lys3). J Biol Chem **267:**15071-15079.

195. **Sodroski, J., W. C. Goh, C. Rosen, A. Dayton, E. Terwilliger, and W. Haseltine.** 1986. A second post-transcriptional trans-activator gene required for HTLV-III replication. Nature **321:**412-417.

196. **Soros, V. B., H. V. Carvajal, S. Richard, and A. W. Cochrane.** 2001. Inhibition of human immunodeficiency virus type 1 Rev function by a dominant-negative mutant of Sam68 through sequestration of unspliced RNA at perinuclear bundles. J Virol **75:**8203-8215.

197. **Strappe, P. M., J. Greatorex, J. Thomas, P. Biswas, E. McCann, and A. M. Lever.** 2003. The packaging signal of simian immunodeficiency virus is upstream of the major splice donor at a distance from the RNA cap site similar to that of human immunodeficiency virus types 1 and 2. J Gen Virol **84:**2423-2430.

198. **Strappe, P. M., D. W. Hampton, D. Brown, B. Cachon-Gonzalez, M. Caldwell, J. W. Fawcett, and A. M. Lever.** 2005. Identification of unique reciprocal and non reciprocal cross packaging relationships between HIV-1, HIV-2 and SIV reveals an efficient SIV/HIV-2 lentiviral vector system with highly favourable features for in vivo testing and clinical usage. Retrovirology **2:**55.

199. **Suhasini, M., and T. R. Reddy.** 2009. Cellular proteins and HIV-1 Rev function. Curr HIV Res **7:**91-100.

200. **Sun, S., V. B. Rao, and M. G. Rossmann.** 2010. Genome packaging in viruses. Curr Opin Struct Biol **20:**114-120.

201. **Swanson, C. M., and M. H. Malim.** 2006. Retrovirus RNA trafficking: from chromatin to invasive genomes. Traffic **7:**1440-1450.

202. **Swanstrom, R., R. C. Parker, H. E. Varmus, and J. M. Bishop.** 1983. Transduction of a cellular oncogene: the genesis of Rous sarcoma virus. Proc Natl Acad Sci U S A **80:**2519-2523.

203. **Teo, I., C. Veryard, H. Barnes, S. F. An, M. Jones, P. L. Lantos, P. Luthert, and S. Shaunak.** 1997. Circular forms of unintegrated human immunodeficiency virus type 1 DNA and high levels of viral protein expression: association with dementia and multinucleated giant cells in the brains of patients with AIDS. J Virol **71:**2928-2933.

204. **Throm, R. E., A. A. Ouma, S. Zhou, A. Chandrasekaran, T. Lockey, M. Greene, S. S. De Ravin, M. Moayeri, H. L. Malech, B. P. Sorrentino, and J. T. Gray.** 2009. Efficient construction of producer cell lines for a SIN lentiviral vector for SCID-X1 gene therapy by concatemeric array transfection. Blood **113:**5104-5110.

205. **Timpe, J., J. Bevington, J. Casper, J. D. Dignam, and J. P. Trempe.** 2005. Mechanisms of adeno-associated virus genome encapsidation. Curr Gene Ther **5:**273-284.

206. **Urcuqui-Inchima, S., C. Patino, X. Zapata, M. P. Garcia, J. Arteaga, C. Chamot, A. Kumar, and D. Hernandez-Verdun.** 2011. Production of HIV particles is regulated by altering sub-cellular localization and dynamics of Rev induced by double-strand RNA binding protein. PLoS One **6:**e16686.

207. **Vargas, J., Jr., G. L. Gusella, V. Najfeld, M. E. Klotman, and A. Cara.** 2004. Novel integrase-defective lentiviral episomal vectors for gene transfer. Hum Gene Ther **15:**361-372.

208. **Voronin, Y. A., and V. K. Pathak.** 2004. Frequent dual initiation in human immunodeficiency virus-based vectors containing two primer-binding sites: a quantitative in vivo assay for function of initiation complexes. J Virol **78:**5402-5413.

209. **Voronin, Y. A., and V. K. Pathak.** 2003. Frequent dual initiation of reverse transcription in murine leukemia virus-based vectors containing two primer-binding sites. Virology **312:**281-294.

210. **Wanisch, K., and R. J. Yanez-Munoz.** 2009. Integration-deficient lentiviral vectors: a slow coming of age. Mol Ther **17:**1316-1332.

211. **Warrier, R., B. R. Linger, B. L. Golden, and R. J. Kuhn.** 2008. Role of sindbis virus capsid protein region II in nucleocapsid core assembly and encapsidation of genomic RNA. J Virol **82:**4461-4470.

212. **Watts, J. M., K. K. Dang, R. J. Gorelick, C. W. Leonard, J. W. Bess, Jr., R. Swanstrom, C. L. Burch, and K. M. Weeks.** 2009. Architecture and secondary structure of an entire HIV-1 RNA genome. Nature **460:**711-716.

213. **Whitcomb, J. M., R. Kumar, and S. H. Hughes.** 1990. Sequence of the circle junction of human immunodeficiency virus type 1: implications for reverse transcription and integration. J Virol **64:**4903-4906.

214. **Wilkinson, K. A., R. J. Gorelick, S. M. Vasa, N. Guex, A. Rein, D. H. Mathews, M. C. Giddings, and K. M. Weeks.** 2008. High-throughput SHAPE analysis reveals

structures in HIV-1 genomic RNA strongly conserved across distinct biological states. PLoS Biol **6:**e96.

215. **Xu, K., H. Ma, T. J. McCown, I. M. Verma, and T. Kafri.** 2001. Generation of a stable cell line producing high-titer self-inactivating lentiviral vectors. Mol Ther **3:**97-104.

216. **Yamashita, M., and M. Emerman.** 2005. The cell cycle independence of HIV infections is not determined by known karyophilic viral elements. PLoS Pathog **1:**e18.

217. **Yamashita, M., and M. Emerman.** 2006. Retroviral infection of non-dividing cells: old and new perspectives. Virology **344:**88-93.

218. **Yamashita, M., O. Perez, T. J. Hope, and M. Emerman.** 2007. Evidence for direct involvement of the capsid protein in HIV infection of nondividing cells. PLoS Pathog **3:**1502-1510.

219. **Yanez-Munoz, R. J., K. S. Balaggan, A. MacNeil, S. J. Howe, M. Schmidt, A. J. Smith, P. Buch, R. E. MacLaren, P. N. Anderson, S. E. Barker, Y. Duran, C. Bartholomae, C. von Kalle, J. R. Heckenlively, C. Kinnon, R. R. Ali, and A. J. Thrasher.** 2006. Effective gene therapy with nonintegrating lentiviral vectors. Nat Med **12:**348-353.

220. **Yedavalli, V. S., and K. T. Jeang.** 2011. Matrin 3 is a co-factor for HIV-1 Rev in regulating post-transcriptional viral gene expression. Retrovirology **8:**61.

221. **Yedavalli, V. S., and K. T. Jeang.** 2010. Trimethylguanosine capping selectively promotes expression of Rev-dependent HIV-1 RNAs. Proc Natl Acad Sci U S A **107:**14787-14792.

222. **Yedavalli, V. S., C. Neuveut, Y. H. Chi, L. Kleiman, and K. T. Jeang.** 2004. Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. Cell **119:**381-392.

223. **Yu, Z., N. Sanchez-Velar, I. E. Catrina, E. L. Kittler, E. B. Udofia, and M. L. Zapp.** 2005. The cellular HIV-1 Rev cofactor hRIP is required for viral replication. Proc Natl Acad Sci U S A **102:**4027-4032.

224. **Zapp, M. L., and M. R. Green.** 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. Nature **342:**714-716.

225. **Zapp, M. L., T. J. Hope, T. G. Parslow, and M. R. Green.** 1991. Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: a dual function for an arginine-rich binding motif. Proc Natl Acad Sci U S A **88:**7734-7738.

226. **Zolotukhin, A. S., A. Valentin, G. N. Pavlakis, and B. K. Felber.** 1994. Continuous propagation of RRE(-) and Rev(-)RRE(-) human immunodeficiency virus type 1 molecular clones containing a cis-acting element of simian retrovirus type 1 in human peripheral blood lymphocytes. J Virol **68:**7944-7952.