The packaging of 7SL RNA by HIV-1

by

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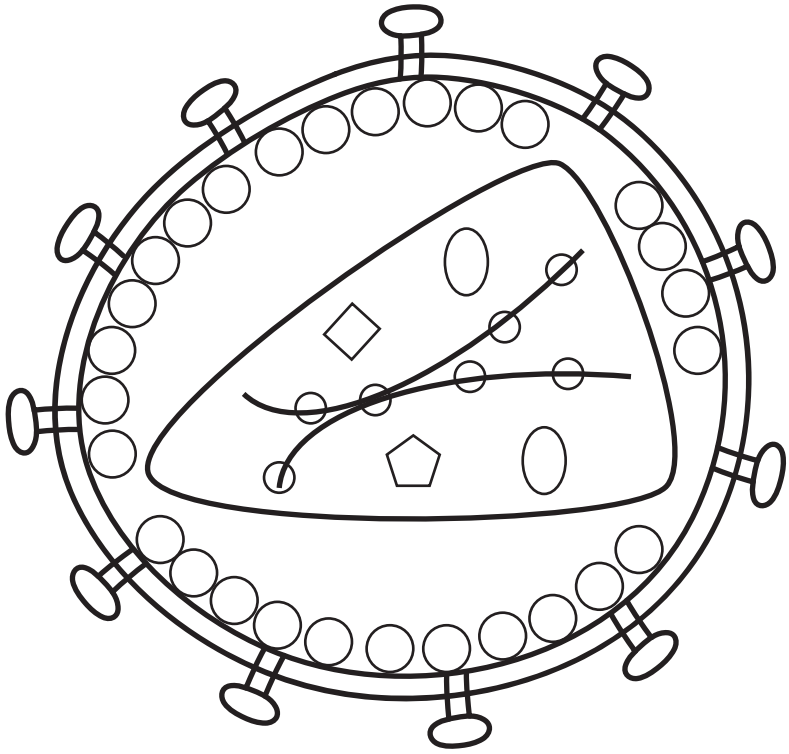

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Steve King performed the S1 nuclease digestion and primer extension shown in Fig. 2-3, and performed the transfection, RNA isolation, and initial northern blot shown in Fig. 2-2.

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Abstract

HIV-1 packages several small cellular RNAs in addition to its genome. Prominent among these is the host noncoding RNA 7SL, of which 12-16 molecules are found per HIV-1 particle. This dissertation examined the packaging of 7SL RNA by HIV-1 to elucidate the *cis*- and *trans*-acting factors that influence 7SL RNA packaging. 7SL consists of two domains, the *Alu* domain and the S domain.

To determine the *trans*-acting packaging factors, minimal virus-like particles were examined for 7SL packaging. It was determined that minimal ΔNC VLPs retain 7SL RNA primarily as an endoribonucleolytic fragment, referred to as 7SL remnant (7SLrem). Nuclease mapping revealed that 7SLrem is a 111-nucleotide internal portion of 7SL, with 5' and 3' ends corresponding to unpaired loops in the 7SL two-dimensional structure. 7SLrem was found in minimal VLPs that contained all Gag proteins except NC, and in minimal VLPs that lacked all of Gag except the C-terminal domain of CA and sp1, indicating that NC protects the *Alu* domain from processing and that CA/sp1 is sufficient for the acquisition of 7SL.

The *cis*-acting regions of 7SL that mediate packaging by HIV-1 were examined by determining the packaging phenotype of 7SL derivatives. Both the *Alu* domain and the S domain were packaged specifically when expressed separately as truncations of 7SL. However, while the *Alu* domain competed with endogenous 7SL

for packaging in proportion to Gag, the S domain was packaged additively, implying that the *Alu* domain is packaged by the same mechanism as endogenous 7SL but that the *Alu* and S domains are packaged via separate mechanisms.

These data suggest that multiple domains of 7SL (*Alu* and S) interact with multiple domains of Gag (CA and NC). The ability of 7SL to interact with multiple domains of Gag suggests that 7SL may play a role in retroviral assembly, possibly by nucleating functional subsets of Gag or by chaperoning Gag to sites of assembly. In addition to the implications for retroviral replication, these data demonstrate the ability to manipulate the cellular RNA content of retroviral virions, which has potential application as a delivery mechanism for modified RNAs such as aptamers.

Chapter One

Introduction

The retrovirus human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS), a devastating disease that has killed over 20 million people worldwide since its discovery in 1983. While HIV-1 has been intensively studied for three decades, much of its basic biology is not yet understood. This dissertation focuses on the factors that influence HIV-1's packaging of a cellular RNA, 7SL.

Retroviruses

Retroviruses are enveloped viruses whose defining characteristic is the reverse transcription of their RNA genome into proviral DNA and the subsequent integration of the proviral DNA into the genome of the host.

Retroviral classification

Retroviruses are classified according to their genomic structure, and are divided into two subfamilies, the orthoretroviruses and the spumaretroviruses. Orthoretroviruses are further divided into six genera: alpha-, beta-, gamma-, delta-, epsilon-, and lentiviruses (61). The first three genera are termed "simple" retroviruses because their genome encodes only three genes, *gag*, *pol*, and *env*, while the latter three genera are

considered to be “complex” retroviruses because they encode additional accessory factors. The work in this thesis will focus on the lentivirus HIV-1. Comparisons will be also made to the gammaretrovirus murine leukemia virus (MLV), which is commonly used as a model retrovirus.

Retroviral genomic structure

Common to all retroviruses are the genes *gag*, *pol*, and *env* (60). Gag and Pol are viral polyproteins, consisting of multiple domains that are cleaved into individual proteins by the viral protease PR during viral maturation (Fig. 1-1a). Gag is the structural polyprotein, whose domains are matrix (MA), capsid (CA), and nucleocapsid (NC). The Gag of HIV also includes spacer protein 1 (sp1) between CA and NC, and spacer protein 2 (sp2) and p6, C-terminal of NC. Pol is the enzymatic polyprotein, whose domains are protease (PR), reverse transcriptase (RT), and integrase (IN). Env, the transmembrane envelope protein found in the virion, is also a polyprotein. Unlike Gag and Pol, Env is cleaved by cellular proteases before viral assembly. The cleavage products of Env are the ectodomain, gp120, and the transmembrane domain, gp41.

Whereas all retroviruses encode the primary genes *gag*, *pol*, and *env*, HIV also codes for six accessory factors: Tat, Rev, Vif, Nef, Vpr, and Vpu (Fig. 1-1b). The regulatory proteins Tat and Rev are necessary for the initial expression of the provirus (158). To facilitate viral replication, mRNAs for Tat and Rev are the first mRNAs to be produced from the proviral DNA and are fully spliced. The transcriptional activator Tat is required for complete transcription from the viral promoter contained within the long

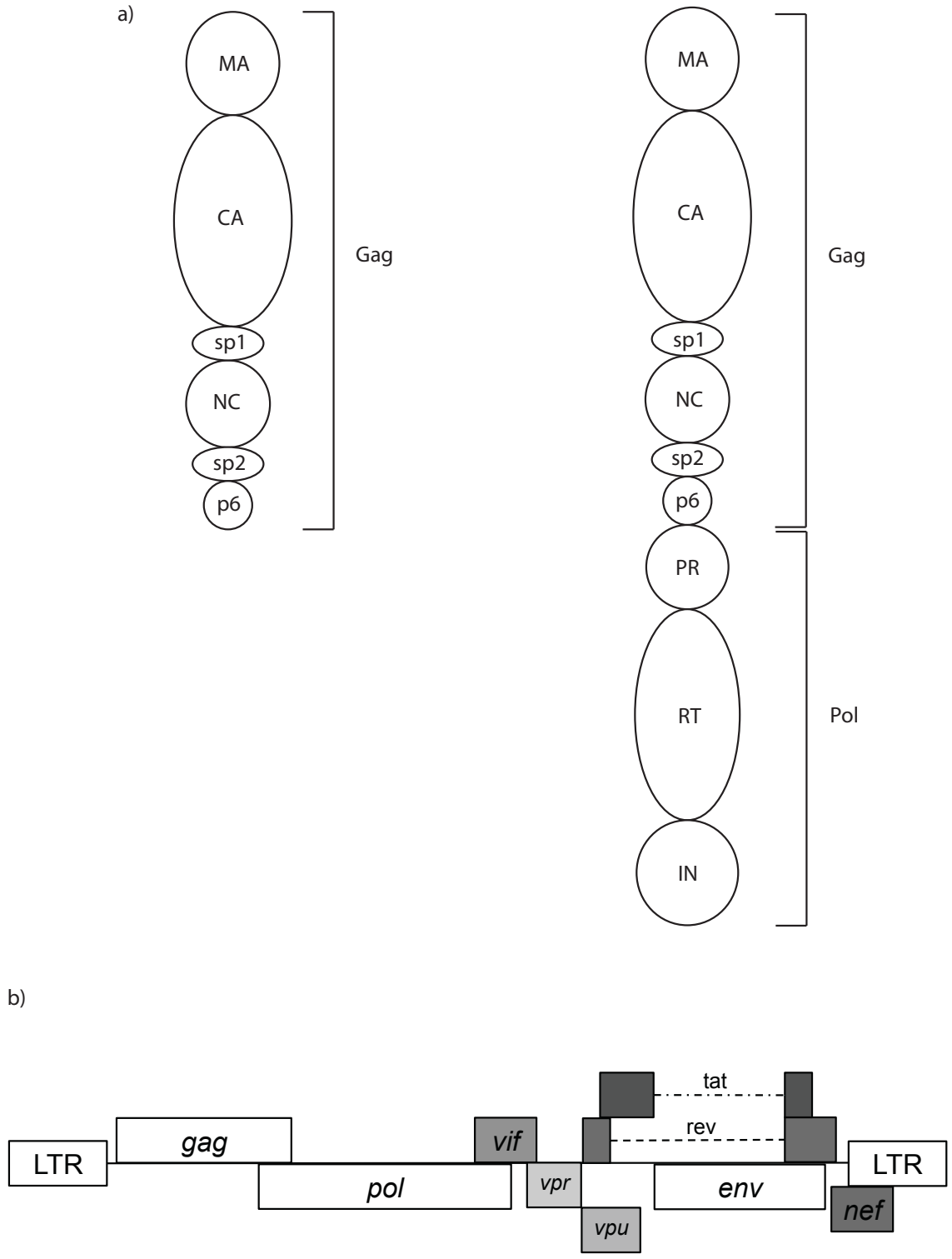

Figure 1-1. HIV proteins and genome. (A) Schematics of HIV-1 Gag (left) and GagPol (right). (B) Schematic of the HIV-1 genome. MA, matrix; CA, capsid; sp1, spacer protein 1; NC, nucleocapsid; sp2, spacer protein 2; PR, protease; RT, reverse transcriptase; IN, integrase.

terminal repeat (LTR). In the absence of Tat, native proviral sequences produce only abortive transcription products (79, 261, 325). Rev binds to a region of viral genomic RNA (gRNA) known as the Rev-responsive element (RRE) and enables the export of gRNA from the nucleus via the CRM1 pathway (92, 165). In the absence of Rev and/or if RRE is mutated, genomic RNA accumulates in the nucleus.

The remaining accessory factors, Vif, Nef, Vpu, and Vpr, are dispensable in many cell culture systems. However, all four appear to be necessary for pathogenesis in the host (4, 114). The viral infectivity factor Vif counteracts the innate antiviral protein APOBEC3G (A3G), a cytosine deaminase that interferes with reverse transcription (231, 274). In contrast, Nef is involved in many of the pathways by which HIV evades cellular-mediated immunity (6, 43): to render infected cells "invisible" to cytotoxic T cells, Nef down-regulates MHC-I molecules from the cell surface. However, because a complete absence of MHC-I molecules from the cell surface is a signal to natural killer (NK) cells, HIV selectively down-regulates the MHC-I proteins HLA-A and HLA-B, but not HLA-C and HLA-E. This prevents lysis by NK cells but still provides some protective effect against cytotoxic T cells (62, 271). Nef may also stimulate the secretion of factors that attract susceptible cells and interfere with the ability of B cells to produce antibodies (255, 294). Viral protein R (Vpr) induces cell cycle arrest in the G2 phase, although the benefit this provides for the virus remains unclear (251). Viral protein U (Vpu) induces the degradation of CD4 (29), thereby reducing superinfection of cells. Vpu also counteracts the innate antiviral protein tetherin (226).

The retroviral life cycle

Retroviral replication can be divided into two phrases: 1) early events, from entry through integration, and 2) late events, from transcription through budding and maturation (Fig. 1-2).

Early events: Entry and reverse transcription

As with all viruses, HIV infection is initiated by the entry of the virus into the cell (323). HIV entry is not pH-dependent, indicating that entry occurs at the plasma membrane (PM) rather than in endosomal compartments (286). However, recent evidence indicates that some entry may occur in endosomal compartments, although this mechanism of entry is still pH-independent (211). Entry begins by the interaction of the transmembrane viral Env protein with the cell-surface immunoglobulin-like CD4. This interaction promotes a conformational change in Env, exposing the co-receptor binding site (173). Depending largely on the identity of two amino acids in the co-receptor binding site, Env can then bind to either of the chemokine receptors CCR5 or CXCR4 (19, 335). Binding to the correct co-receptor triggers a further conformational change in Env and results in the fusion of the viral and cellular membranes and the delivery of the viral core to the cytoplasm of the newly infected cell (207).

The viral core is then transported from the plasma membrane to the nuclear membrane. During this time, the majority of the viral proteins that make up the viral core are shed in a process called uncoating, although exactly what this process involves, and when and where it occurs, remain unclear (5).

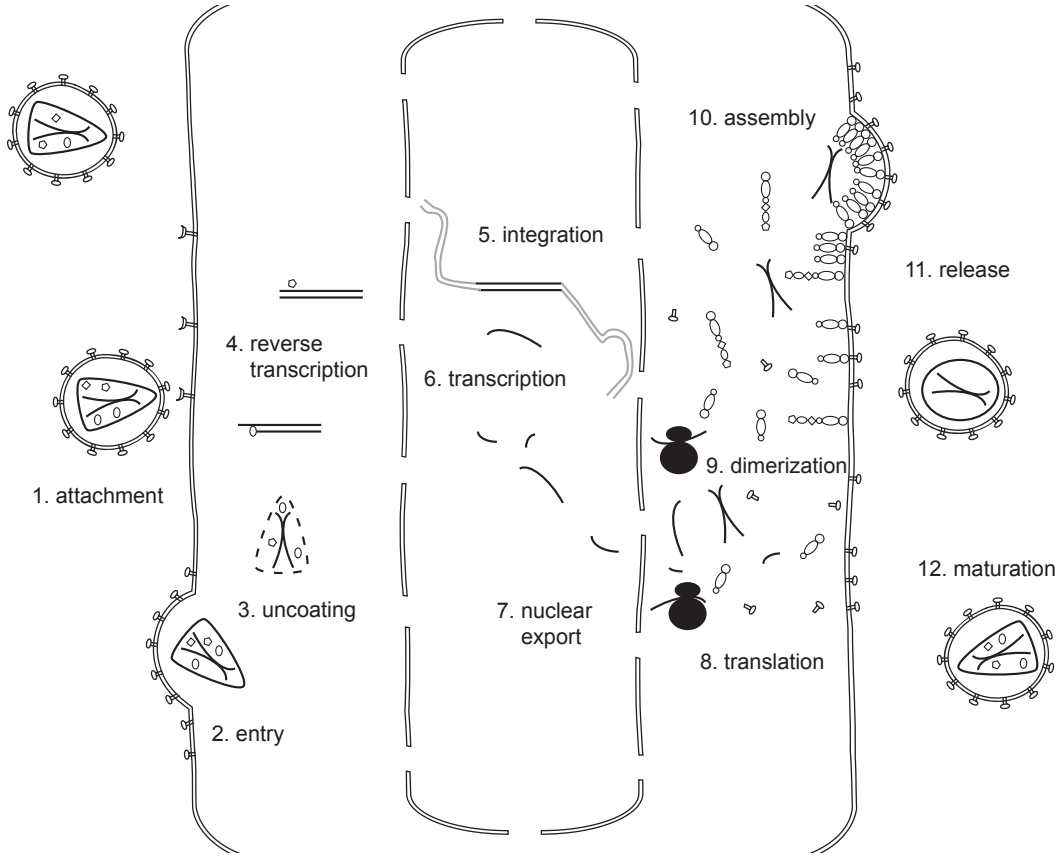

Figure 1-2. The retroviral life cycle.

After delivery to the cytosol and prior to transport across the nuclear membrane, reverse transcription occurs (Fig. 1-3) (298). Reverse transcription is thought to be initiated by the sudden appearance of non-limiting amounts of deoxynucleotides upon entry of the core to the cytosol (216). Successful reverse transcription is also dependent on successfully regulated uncoating – that is, uncoating must not be too early or too late, or involve the loss of too many capsid proteins or too few. Mutations in Gag that increase or decrease the stability of capsid during uncoating block retroviral replication at the reverse transcription step (313). The cytoplasmic antiviral protein TRIM5α interferes with uncoating by interacting with capsid and likewise blocks replication at the reverse transcription step (291).

Reverse transcription is carried out by the viral enzyme reverse transcriptase (RT), a DNA polymerase that can use either RNA or DNA as its template (141, 180). RT also contains an RNase H domain and degrades the RNA component of an RNA:DNA hybrid (285). Like all DNA polymerases, RT requires a primer, which is a specific tRNA packaged by the virion during assembly (199). Reverse transcription initiates at the primer binding site, using the tRNA as a primer, and then continues to the 5' end of the genomic RNA (7). Because the RNase H activity of RT degrades the RNA template as DNA synthesis progresses, the product of this first step of reverse transcription is single-stranded, negative sense DNA known as minus strand strong stop DNA. Using the homology of the R regions, RT performs the first forced template switch, from the 5' to the 3' end of the RNA (14).

Reverse transcription continues until the 5' end of the RNA is reached, a process that takes several hours. RNase H continues to degrade the RNA template during

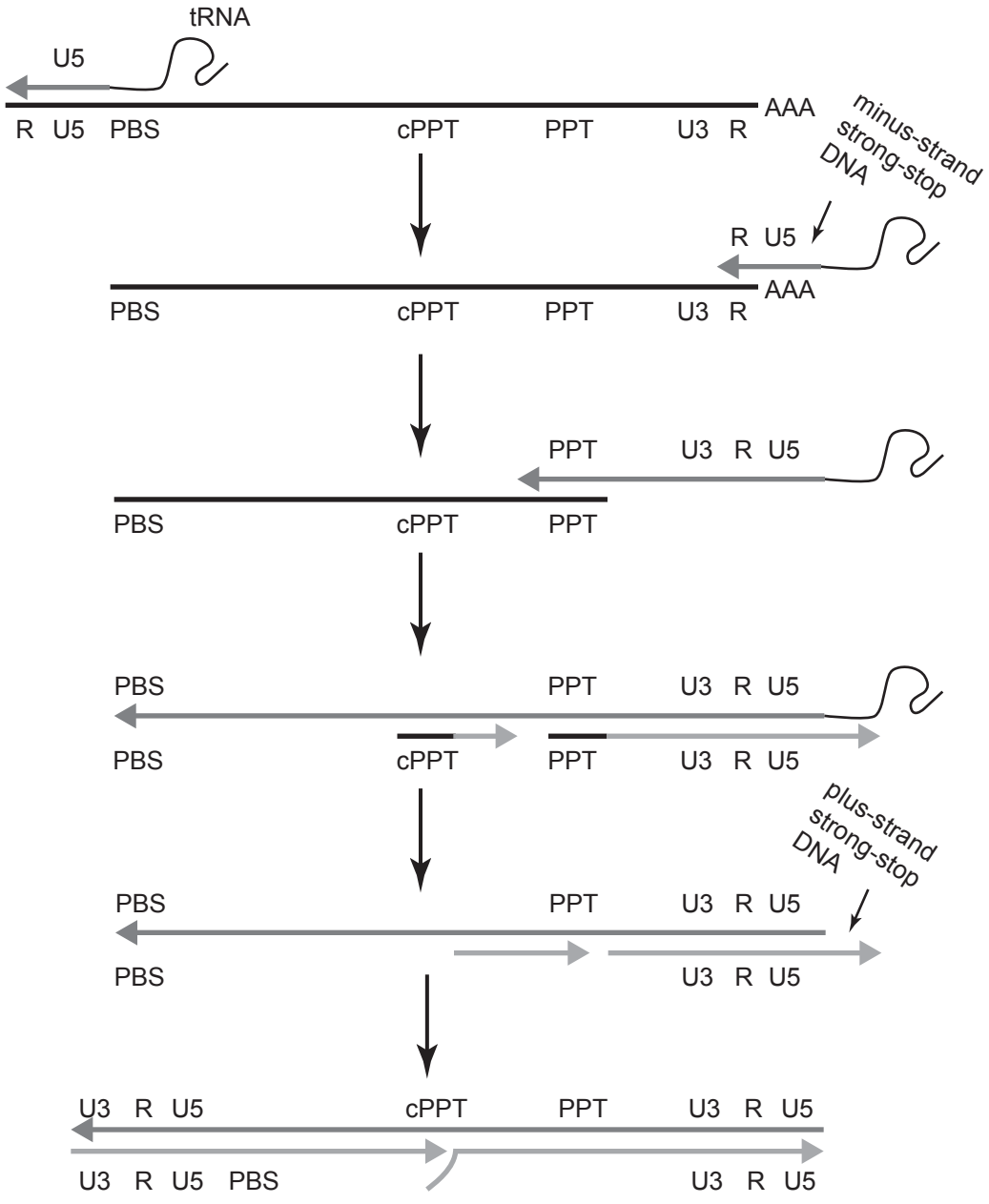

Figure 1-3. Reverse transcription. Black, RNA; dark grey, minus strand DNA; light grey, plus strand DNA. (Adapted from reference 298).

transcription. However, two purine-rich regions of RNA known as the polypurine tracts (PPT) are resistant to RNase H degradation. Two additional RT molecules use the PPT RNA as primers to initiate the transcription of the second, positive sense strand of DNA (48, 49). Transcription continues through 18 bp of the primer tRNA, which is subsequently degraded because it is RNA. The DNA strand thus produced (the 3' end of which is single-stranded due to digestion of the primer tRNA) is the plus strand strong stop DNA. After completion of the minus strand DNA, RT mediates the second forced template switch, using the homology of the primer binding site (16). Transcription continues until the plus strand DNA is completed, yielding double-stranded proviral DNA (108).

After reverse transcription, proviral DNA must be imported into the nucleus for integration into the host genome. Unlike gammaretroviruses such as MLV, HIV can infect non-dividing cells, implying transport across an intact nuclear membrane (35, 259, 318). Evidence suggests that the unit that is transported across the nuclear membrane is the pre-integration complex (PIC), consisting of proviral DNA, MA, RT, and IN (84, 90, 143). The PIC is formed after reverse transcription is completed and is transported across the nuclear membrane by cellular factors, although the exact mechanism by which this occurs remains unknown (210).

Integration of proviral DNA into the host chromosome is mediated by IN and occurs in a single concerted event (53). Evidence suggests that the integration site is essentially random, although HIV exhibits a preference for transcriptionally active DNA (133, 185). To facilitate integration into transcriptionally active regions of DNA, IN uses the cellular protein LEDGF to tether the integration complex to chromatin (59, 189, 276).

Integration is irreversible, so that infection by one virus yields a potential reservoir for new virus that lasts until the death of the cell (or the host). The irreversibility of integration and the ability of an integrated provirus to remain latent until transcriptional activation is one of the primary difficulties encountered when searching for a "cure" for HIV infection.

Late events: Transcription, translation, and assembly

To complete the infection cycle, the integrated provirus must be transcriptionally activated. Although the initiation of transcription from the LTR promoter is highly efficient, initial transcription is abortive due to inefficient elongation. However, short, multiply spliced RNAs coding for Tat and Rev are produced (158, 341). Transcription remains at this level until Tat is translated and imported into the nucleus. Tat binds to a region of the nascent mRNA immediately 3' of the HIV promoter termed TAR. In conjunction with multiple cellular co-factors, Tat stabilizes the elongation complex, enabling efficient transcription of the unspliced gRNA/GagPol mRNA and the alternatively spliced Env, Vif, Nef, Vpr, and Vpu mRNAs (79, 217).

Because the RNA that serves as both the genomic RNA and the mRNA for Gag and GagPol is unspliced, HIV must circumvent the RNA quality control pathways that typically retain and degrade unspliced and incompletely spliced RNA in the nucleus. The regulatory protein Rev enables this circumvention by binding to a region of HIV RNA known as the Rev-responsive element (RRE) and facilitating the export of the intron-containing RNA from the nucleus by interacting with the nuclear export factor CRM-1 (69, 125, 165, 200, 227, 254). Proviruses that have been modified so that their RNA

product uses an alternative nuclear export pathway can generate viral proteins but do not efficiently form viral particles in tissue culture. This suggests that proper trafficking of unspliced HIV RNA through the CRM-1 pathway is crucial not only for efficient nuclear export but also for later trafficking and assembly of Gag (149, 150, 274).

After export from the nucleus, viral mRNA is translated. The typical ribosomal scanning mechanisms used for the translation of cellular mRNAs are ineffective on the unspliced viral mRNA/gRNA because of the multiple tertiary structures in the 5' region of the RNA, including TAR, PBS, and Ψ. To overcome this difficulty, HIV gRNA/mRNA likely encodes an internal ribosome binding site (IRES) and employs multiple cellular co-factors to enhance translation (27). Approximately 5% of the time during the translation of Gag, a -1 ribosomal frameshift occurs near the end of the Gag reading frame, resulting in a shift into the Pol reading frame. This causes around 1 in every 20 proteins produced to be the GagPol fusion protein (Fig. 1-1) (32).

Subsequent steps in the viral life cycle involve the trafficking of the viral proteins and RNA to the plasma membrane, the assembly of the viral particle, the budding of the virus from the host cell, and finally, the maturation of the viral particle into its fully infectious form. These steps will be discussed in more detail below.

Retroviral assembly

In brief, HIV assembly can be summarized in four steps: 1) targeting of Gag and gRNA to the plasma membrane (PM); 2) binding of Gag to the PM; 3) Gag multimerization and gRNA encapsidation; and 4) budding and release (100, 101).

Domains of Gag

The structural polyprotein Gag is necessary and sufficient to assemble virus-like particles (VLPs) both *in vitro* and *in vivo* (40, 41, 65, 106). Gag is composed of six domains that are subsequently cleaved into independent proteins during virion maturation (Fig. 1-1) (270). The following paragraphs focus on the functions of these domains as parts of the Gag polyprotein during assembly rather than on their roles as independent proteins post-maturation.

The N-terminal domain of Gag is the matrix (MA) domain, which is responsible for targeting Gag to the plasma membrane (PM). The N-terminus of MA is post-transcriptionally modified with a myristate moiety that is sequestered in a hydrophobic region of the protein until MA binds the PM (112, 295). MA also contains a positively charged highly basic region (HBR) and a conserved region from amino acids 84-88, both of which are required for proper membrane targeting (96, 130, 239, 240).

The central region of Gag consists of capsid (CA) and spacer protein 1 (sp1), which together are responsible for the Gag/Gag lattice interactions that are necessary for Gag multimerization (100, 170, 272). CA consists of an N-terminal domain (CA-NTD) and a C-terminal domain (CA-CTD), which are joined by a flexible linker. sp1 is also joined to CA-CTD by a flexible linker. Within the CA-CTD lies the major homology region (MHR), which is highly conserved across all retroviruses and is necessary for proper particle formation and morphogenesis (82, 201). Together, CA and sp1 mediate the formation of the hexamers of Gag that are the functional unit of the Gag lattice, although only the CA-CTD and sp1 are absolutely required for particle production (1, 28, 326).

The nucleocapsid (NC) domain is connected to sp1. NC contains multiple RNA-binding motifs, including two zinc finger (ZF) motifs that interact specifically with gRNA and several basic regions that bind non-specifically to RNA. NC is responsible for recruiting the genomic RNA to the sites of assembly and for encapsidating gRNA during particle formation (67, 257).

Spacer protein 2 (sp2) and p6 are C-terminal of NC. Within p6 are two so-called "late domains", the PT/SAP domain and the YPX_nL domain, which promote budding and release of the immature virion by recruiting the cellular ESCRT machinery (73, 95). p6 also mediates Vpr incorporation (11, 267).

The architecture of the virion

To understand viral assembly, the structure of the immature virion must first be understood (101). Fig. 1-4 shows a schematic of the immature and mature virion. Immature retroviral particles are spherical shells of the Gag protein surrounded by a lipid bilayer derived from the host cell plasma membrane (324). Interestingly, the shell of Gag is incomplete when the virus buds from the cell, although the reason for this remains unknown (33, 72, 326). The N-terminal MA domain of Gag is bound to the viral membrane, while the C-terminal p6 domain is at the center of the virion. The viral genomic RNA is condensed at the core of the virion by interactions with the NC domain. Also in the core of the virion are the Pol regions of the GagPol fusion proteins, as well as multiple cellular factors that are packaged by the virus. Env exists as a trimer of heterodimers (gp120 and gp41) tethered to MA and protruding through the viral membrane.

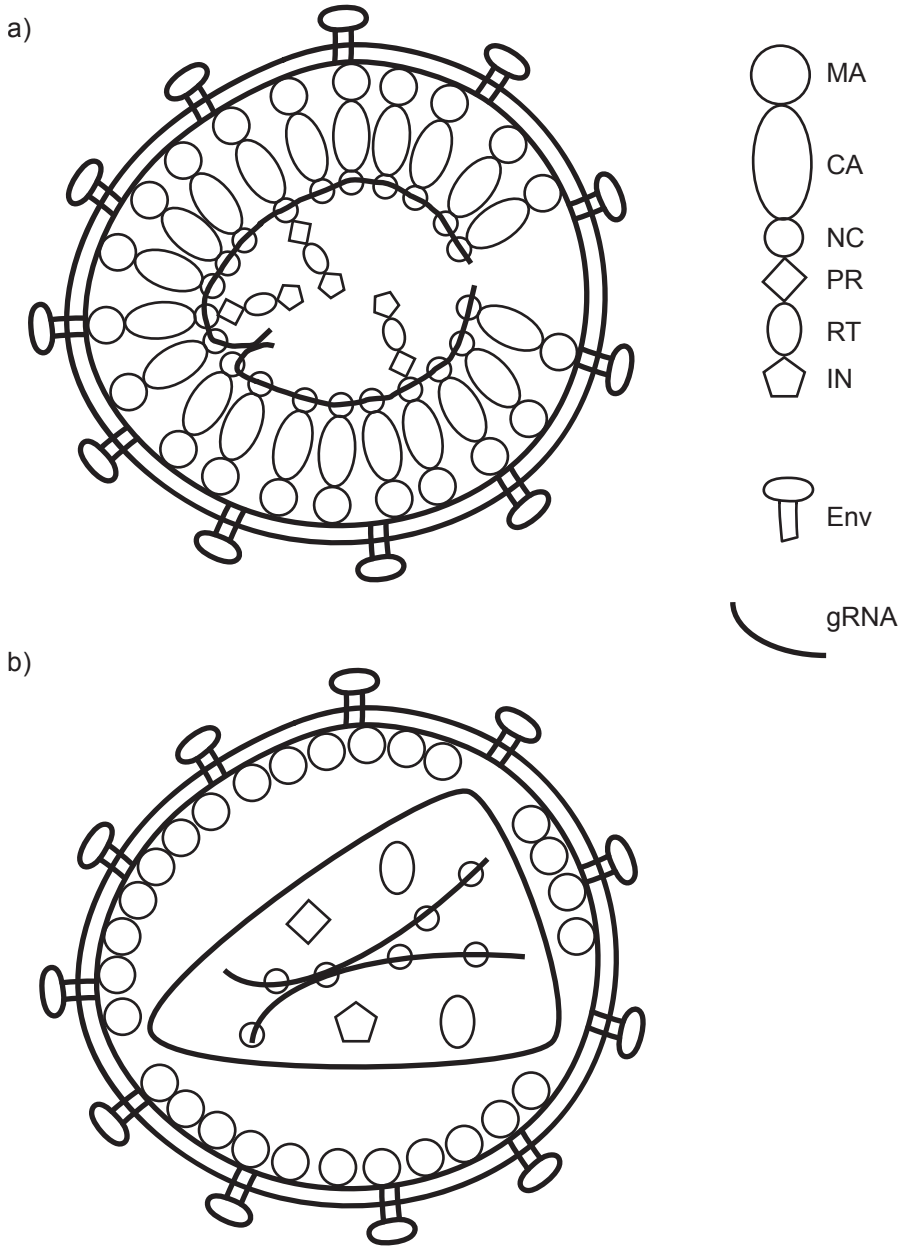

Figure 1-4. The HIV virion. Schematics of the immature (A) and mature (B) HIV-1 virion. The conical core of the mature virion consists of capsid. MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; IN, integrase; Env, envelope; gRNA, genomic RNA.

After release, the viral protease (PR) domain of Pol dimerizes and undergoes autoproteolysis to release the functional protease (169). Further cleavage of Gag and GagPol occurs in a temporally defined sequence. Mutations in Gag that alter the order in which Gag is cleaved produce non-infectious virus, likely due to misfolding of the liberated proteins (219, 249). Cleavage promotes the condensation of CA into a highly ordered conical core composed of CA hexamers. However, to maintain the proper curvature of the lattice, 12 pentamers are required (5 at the narrow end of the cone and 7 at the wide end) (186). Within the CA capsid remain the gRNA (now further condensed with NC), the viral enzymes RT and IN, and multiple cellular factors. MA remains as a spherical shell just under the lipid bilayer.

Intracellular Gag trafficking

Viral assembly occurs at the plasma membrane, requiring the movement of Gag from cytosolic ribosomes to the outer boundary of the cell (13, 55, 88, 222). Two classes of proteins normally involved in vesicle trafficking have been implicated in Gag trafficking: clathrin-associated heterotetrameric adaptor protein (AP) complexes and ADP-ribosylation factor (Arf) proteins. AP-1 is normally involved in the bidirectional transport of vesicles between the trans-Golgi network and endosomes, while AP-3 is normally involved in the transport of vesicles from early endosomes and the TGN to late endosomes, multivesicular bodies, and lysosomes (223, 234). Both AP-1 and AP-3 interact with the MA domain of Gag. Depletion of both AP-1 and AP-3 leads to altered intracellular localization of Gag and reduced particle production (42, 81, 103). Arf proteins are responsible for trafficking GGA (Golgi-localized, γ-ear containing, Arf-

binding) proteins to the plasma membrane (68, 109, 156). Overexpression of GGA proteins reduces HIV-1 particle production, while depletion of GGA proteins enhances particles production. Depletion of Arf proteins also leads to reduced viral release (153, 154). Together, these data suggest that HIV Gag competes with GGA for Arf binding. Further details on how the Arf and AP proteins mediate Gag trafficking remain to be elucidated.

Intracellular movement also requires components of the cytoskeleton. In the cell, microtubules mediate long-range transport, while actin filaments mediate short-range transport. Microtubules, therefore, would be expected to be involved in the transport of Gag to the PM, although actin has been reported to be involved at the site of viral assembly (110). Inhibition of tubulin remodeling, necessary for microtubule-based transport, disrupts Gag trafficking to the PM (151). Furthermore, Gag interacts with the kinesin KIF4, and depletion of KIF4 disrupts Gag trafficking and inhibits particle production (204, 296). Because kinesins are the microtubule-based motor proteins responsible for the trafficking of cargo towards the PM, these data suggest that Gag is transported from its sites of translation to sites of assembly by employing the microtubule-based transport machinery.

Targeting of Gag to the PM and Gag's interactions with lipids

HIV does not assemble on the membranes of intracellular compartments such as endosomes, instead, it preferentially assembles on the plasma membrane or, in macrophages, on invaginations of the plasma membrane (74, 319). This targeting ability maps to the MA domain of Gag, as Gag molecules lacking MA exhibit promiscuous

membrane targeting (284). Specific mutations to the HBR or amino acid residues 84-88 of MA mistarget Gag to the membranes of late endosomes, indicating that it is these regions specifically that are involved in PM targeting (96, 130, 239, 240). However, in the context of ΔMA Gag, only the N-terminal six amino acids (including the myristylation signal) are required for efficient PM targeting, implying that the HBR and aa 84-88 are necessary for PM targeting only in the context of full-length Gag (1, 247).

The HBR of MA targets Gag to the PM by interacting specifically with the lipid phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P_2]. PI(4,5)P_2 is a type of lipid known as a phosphoinositide. PI(4,5)P_2 localizes to the inner leaflet of the PM, which is also the assembly site for HIV (314). Two lines of evidence initially led to the hypothesis that PI(4,5)P_2 is involved in HIV assembly: 1) when PI(4,5)P_2 is depleted from the cell by overexpression of polyphosphoinositide 5-phosphatase IV, Gag either mislocalizes to perinuclear membranes or remaines cytoplasmic (56, 238); and 2) when PI(4,5)P_2 is relocalized to an intracellular compartment by expression of a constitutively active form of Arf6, Gag localizes to this new compartment instead of the PM (238).

Furthermore, the mechanism by which MA binds PI(4,5)P_2 has been elucidated. Prior to binding PI(4,5)P_2, the myristate moiety on the N-terminus of MA is bound in a hydrophobic pocket. The phosphoinositol headgroup of PI(4,5)P_2 binds to the HBR of MA, which shows high specificity for PI(4,5)P_2 over other phosphoinositides (264, 275). The unsaturated fatty acid chain of PI(4,5)P_2 binds to a hydrophobic groove adjacent to the sequestered myristate, prompting a conformational change that exposes the myristate and allows it to insert into the PM (264). Gag is therefore tethered to the PM by two saturated fatty acids: the myristate and the saturated fatty acid side chain of PI(4,5)P_2.

Acquisition and encapsidation of the viral RNA

HIV virions are highly enriched in their genomic RNA relative to their concentration in the cell. While gRNA consists of only ~3% of the RNA content of the cell, it is over 50% of the RNA content of the virion (21, 263). This enrichment alone would indicate that gRNA packaging is non-random. Further supporting this finding, the determinants of packaging have been identified for both genomic RNA and the Gag protein (67, 182). The region of genomic RNA responsible for mediating its packaging is known as the *p*ackaging *si*gnal, or psi (Ψ); mutations in the Ψ site of gRNA severely reduce the packaging efficiency of the genome (3, 183). Similarly, the region of Gag responsible for mediating gRNA packaging is the NC domain; ΔNC VLPs and VLPs containing Gag with specific mutations in the NC domain also contain severely reduced amounts of genome (3, 77, 241). The NC domain of Gag is not only highly specific for RNAs that contain a Ψ site over those that do not, but also NCs are highly specific for the Ψ site of their own species. Chimeric Gags in which the NC domain has been replaced by an NC domain of another retrovirus preferentially package RNAs containing the Ψ site of the same retrovirus species as the NC domain (20, 339). The ability of HIV-1 NC to specifically recognize Ψ maps to two zinc finger (ZF) motifs with the sequence C-X_2-C-X_4-H-X_4-C (the "CCHC" motifs) (3). Mutations to any of the zinc-coordinating cysteines or histidines result in the production of viral particles that package significantly less genome (3). Similarly, treatment of viruses with zinc chelators render the virus non-infectious (258).

Unlike simple retroviruses such as ALV and MLV, whose Ψ sites are small and well-defined, the Ψ site of HIV covers the entire 5' UTR and likely extends into the *gag* coding region (188). Mutational analysis of the HIV Ψ site has been hampered by the highly structured character of the 5' UTR – small mutations affect packaging, but it is often difficult to determine exactly what effects on the structure of the RNA these mutations have (77, 176, 192). However, recent studies have narrowed down the region of the 5' UTR necessary for dimerization and packaging to a 159 nt sequence (128, 191).

Ψ is known to overlap the dimerization initiation site, and multiple lines of evidence suggest that gRNAs are packaged as pre-formed dimers (215). Mutations that affect dimerization also reduce packaging (135). gRNA is found in viral particles as a dimer, even when gRNA packaging is significantly reduced, and nonviral RNAs to which Ψ sites have been added are also packaged as dimers (51, 131). In MLV, dimerization has been shown to expose sites that undergo high affinity interactions with NC (212). Recent evidence indicates that HIV employs a similar mechanism, although this remains an active area of investigation (128).

NC is not the only region of Gag known to interact with RNA. The HBR of MA can also bind nucleic acid (243). When particles are assembled *in vitro* out of Gag and nucleic acid, they are 25-30 nm in diameter, rather than the ~120 nm diameter of authentic immature particles (40). These smaller particles are consistent with rods of Gag being in a "bent over" conformation, with both NC and MA in contact with the nucleic acid. Biochemical analysis provides evidence that Gag in solution is in this same "folded-over" conformation, and particles formed *in vitro* are the correct size if polycationic lipids are added along with nucleic acid to Gag (39, 65, 71). MA appears to have higher

specificity for lipids than RNA; and lipids, especially $PI(4,5)P_2$, compete directly for binding to the HBR of MA (57). These data have led to a model in which Gag contacts the gRNA with both its NC and MA domains until it reaches the PM, at which point $PI(4,5)P_2$ successfully competes for MA binding with RNA, and the tethering of Gag to the PM is achieved via $PI(4,5)P_2$ and the myristate moiety. It has been hypothesized that this mechanism helps prevent the non-specific interaction of MA with lipids and the resulting promiscuous membrane targeting of Gag (57).

The exact cellular location of the first interaction between Gag and gRNA remains unknown. However, recent evidence indicates that gRNA is recruited to the PM as a pre-formed dimer, likely in complex with a few Gag molecules; this indicates that the initial interaction between Gag and gRNA occurs before the targeting to the site of viral assembly (51, 155). There are two models that describe the pathway by which a retroviruses can select its genome: 1) in the "one pool" hypothesis, the same mRNA that is used to translate Gag molecules can be acquired by those very same Gag molecules; and 2) in the "two pool" hypothesis, one population of viral RNAs leaving the nucleus becomes gRNA without being translated, and a separate population of viral RNAs is translated without being packaged. Various lines of evidence suggest that MLV employs the two-pool model, while the same tests suggest that HIV employs one pool (51, 93, 94, 215). However, *cis* packaging of gRNA is favored rather than obligatory, as Gag proteins produced from a Ψ(-) RNA can package a co-expressed Ψ(+) gRNA that does not produce Gag (128).

RNA as a structural component of the retroviral particle

In addition to the role of gRNA as the viral genome, RNA – but not necessarily gRNA – has been proposed to serve a structural role during assembly. Supporting this hypothesis is the necessity of using RNA to prime assembly *in vitro*: purified Gag molecules are soluble in solution, but form VLPs upon the addition of nucleic acid (40, 41, 65). Further evidence of the role of RNA in assembly comes from analysis of NC mutants. Deletion of the NC domain reduces the production of intact particles by about 40-fold. However, this phenotype is rescued if the NC domain is replaced by a domain capable of forming protein-protein interactions such as a leucine zipper (LZ) motif (1, 65, 340). These data have been used to propose a model in which the binding of RNA by the NC domain promotes the oligomerization of Gag.

The identity of the nucleic acid used to promote the oligomerization of Gag remains unclear. *In vitro*, the requirement for nucleic acid to prime assembly is non-specific as to the identity of that nucleic acid: both RNA and DNA can prime assembly equally well, and nucleic acids can be as short as 20-40 nucleotides with no appreciable loss in particle production (40, 195, 196). It can be assumed that Gag is more selective *in vivo* because the RNA content of a viral particle produced in a cell is non-random (see below), and wild-type particles do not contain any DNA.

The gRNA is a candidate for playing a role in assembly, as it is known to interact with the majority of Gag molecules present in the virion through the NC domain. Indeed, when viral particles are prevented from packaging gRNA because of mutations in the Ψ site, they package comparable amounts of cellular mRNAs, implying that gRNA must be replaced by RNA of similar characteristics for efficient particle production (263).

However, genomic RNA is likely not the only RNA serving a structural role during assembly: mutations in the ZF motifs of NC, which mediate specific encapsidation of gRNA, do not affect particle production, while mutations in the basic region of NC, which recognizes RNA non-specifically, reduce particle yield by ~10-fold (58, 308). These findings raise the possibility that alternative (i.e., cellular) RNAs are employed by the retrovirus to facilitate Gag multimerization.

Cellular RNAs in retroviruses

Early sedimentation studies of avian retroviruses revealed that retroviruses package a number of small cellular RNAs in addition to the ~70S viral RNA, data that have since been corroborated in other retroviruses (23, 24, 89). Indeed, up to 30% of the RNA in a retroviral particle is of cellular origin (188). The majority of these cellular RNAs fall between 4S and 8S on a sedimentation gradient. The following subsections discuss the cellular RNAs found in retroviral particles in greater detail.

tRNAs

A large amount of the 4S RNA found in retroviral particles is tRNA (23, 89, 148). As mentioned previously, retroviruses use cellular tRNA to prime reverse transcription (199). The tRNA used differs among retroviruses; HIV-1 and other lentiviruses use the third isoform of the lysyl-tRNA, $tRNA^{Lys,3}$ (166). Primer tRNAs are selectively packaged by retroviruses, with their relative concentration in the virion exceeding their relative concentration in the cell. HIV packages about 20 molecules of $tRNA^{Lys}$ per virion. About 8 molecules in an HIV virion are $tRNA^{Lys,3}$, and the remaining 12 molecules are

$tRNA^{Lys1,2}$, a distribution that reflects the distribution of lysyl-tRNA isoforms in the cell (98, 139, 148).

Primer tRNAs are packaged as part of a complex that includes RT, NC, and gRNA. Pol, and specifically the RT domain of Pol, is required for $tRNA^{Lys}$ incorporation (198). Surprisingly, despite the evidence that primer tRNA is already bound to gRNA in the proper position to initiate reverse transcription in the mature virion, and despite data showing that changes in the sequence of the primer binding site of gRNA can change the identity of the packaged primer tRNA, gRNA is not required for $tRNA^{Lys}$ encapsidation (198). The NC domain of Gag and GagPol is also not required (137).

A major determinant for the packaging of $tRNA^{Lys}$ is the simultaneous packaging of the cellular protein responsible for aminoacylating $tRNA^{Lys}$, lysyl-tRNA synthetase (LysRS). LysRS is selectively packaged by HIV via interactions with the CA-CTD (146, 167, 168). Although GagPol is required for packaging of $tRNA^{Lys}$, Gag alone is sufficient for LysRS encapsidation (45). Nevertheless, LysRS appears to be directly responsible for the acquisition of $tRNA^{Lys}$: overexpression of LysRS in cells producing HIV-1 results in both an increase in the packaged LysRS and a proportional increase in packaged $tRNA^{Lys}$; knockdown of LysRS by RNAi in HIV-producing cells results in a decrease in the packaged LysRS and a proportional decrease in packaged $tRNA^{Lys}$ (98, 119).

mRNAs

Cellular mRNAs are not highly packaged by HIV unless the viral gRNA is not packaged due to mutations in the Ψ site, as described above. Even when cellular mRNAs

are packaged as a replacement for gRNA, the identity of the packaged mRNA is random (263).

Pol III products

The remaining packaged cellular RNAs have only begun to be characterized. In previous work, the cellular RNA content of MLV was examined and found to be primarily products of RNA polymerase III (Pol III) (236). Unlike Pol II, which primarily produces mRNA, Pol III produces small, non-coding RNAs that primarily have structural or enzymatic functions in the cell (244, 321). MLV was found to selectively package the spliceosomal RNA (snRNA) U6, the Y RNAs mY1 and mY2, the 5S ribosomal RNA, 7SL RNA, and the murine 7SL derivative B1 (236). The 5.8S and 18S ribosomal RNAs were found to be much less enriched than 5S rRNA, and the other spliceosomal RNAs, U1, U2, U4, and U5, were also found to be much less enriched than U6 snRNA. Interesting, mY1 and mY3 were found to be packaged by MLV even when the retrovirus was expressed in Ro-knockout cells; Ro is a protein that stabilizes Y RNAs, and Y RNAs in Ro-knockout cells are rapidly degraded, presumably in the nucleus (104). These data indicate that Y RNAs are acquired by MLV early in their biogenesis.

All of these selectively packaged cellular RNAs are Pol III products. In fact, the 5S rRNA and the U6 snRNA are the only rRNA and snRNA produced by Pol III; 5.8S and 18S rRNAs are produced by Pol I, and the remaining snRNAs (U1, U2, U4, and U5) are produced by Pol II (244). These data suggest a selection bias towards the packaging of Pol III products. However, it is unclear whether this finding is due to a shared feature of Pol III products – for example, their poly(U) 3' ends or a shared trafficking pathway –

or due to a more general characteristic of these RNAs, such as their highly ordered three-dimensional structures.

Further work on the RNA content of HIV revealed both differences and similarities with MLV. The RNA profile of HIV is also highly biased toward Pol III products, and many Pol III products are found in both viruses. For example, HIV also packages 7SL, U6, and 5S rRNA (77, 135, 237, 263, 302). However, wild-type HIV does not package hY1 and hY3 in appreciable amounts, nor does it package the human 7SL derivative scAlu (302, 309 and unpublished data). Further detailed work on the RNA profile of HIV remains to be performed.

Of particular interest in the list of selectively packaged cellular RNA is the 7SL RNA. 7SL is found not only in HIV and MLV, but also in Rous sarcoma virus (RSV), avian myeloblastosis virus (AMV), Visna virus (VV), feline infectious leukemia virus (FeLV), and equine infectious anemia virus (EIAV) (24, 31, 50, 83, 187). 7SL was found to be packaged at 3-4 molar excess over genomic RNA in MLV, and 6-7 molar excess over genomic RNA in HIV (236, 237). In other words, between 12 and 14 copies of 7SL are found per virion in HIV. The commonality of selective 7SL packaging across classes of retroviruses, the abundance of 7SL within the virion, and the structural role of 7SL in the cell (see below) all make 7SL a prime candidate for a proposed role in retroviral assembly.

7SL

7SL is the RNA component of the signal recognition particle (SRP), which targets nascent membrane and secretory polypeptides to the endoplasmic reticulum (ER). In

cells, translation initially occurs on free ribosomes. However, if the protein undergoing translation is destined to become a transmembrane or secreted protein, the ribosome/mRNA/polypeptide complex must be transported to the ER early in translation so that the growing polypeptide can be threaded into or through the ER membrane (66, 193). Both elongation arrest of the nascent polypeptide and targeting of the ribosomal complex to the ER are mediated by the SRP, which is recognized by the SRP receptor (SR) on the ER membrane.

The eukaryotic SRP is composed of the 7SL RNA and six proteins: SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72 (Fig. 1-5). 7SL RNA is divided into three regions: the left-hand *Alu* domain, the right-hand S domain, and the linker region, which separates the two. The *Alu* domain is bound by the SRP9/14 heterodimer (140, 292, 315, 317), while the S domain is bound by the SRP68/72 heterodimer and the proteins SRP19 and SRP54 (208, 242, 265, 277).

Genesis of the SRP begins in the nucleolus, where 7SL is transcribed (144, 252). All SRP proteins except SRP54 have nucleolar localization signals, and bind to 7SL in a prescribed manner: first SRP9/14, then SRP68/72 and SRP19 (52, 118, 253, 316). 7SL is therefore rarely, if ever, found in the cytoplasm without its accompanying SRP proteins (30). After these proteins have bound, the incomplete SRP is translocated out of the nucleus, and SRP54 binds in the cytoplasm (197). It has been amply demonstrated that SRP54 can only bind to 7SL after a conformation change of the RNA that is caused by the binding of SRP19 (113, 121, 172, 242, 268, 322, 331).

Once fully formed, the SRP is capable of targeting polypeptide/ribosome complexes to the ER. Proteins destined for transport to the ER via the SRP pathway

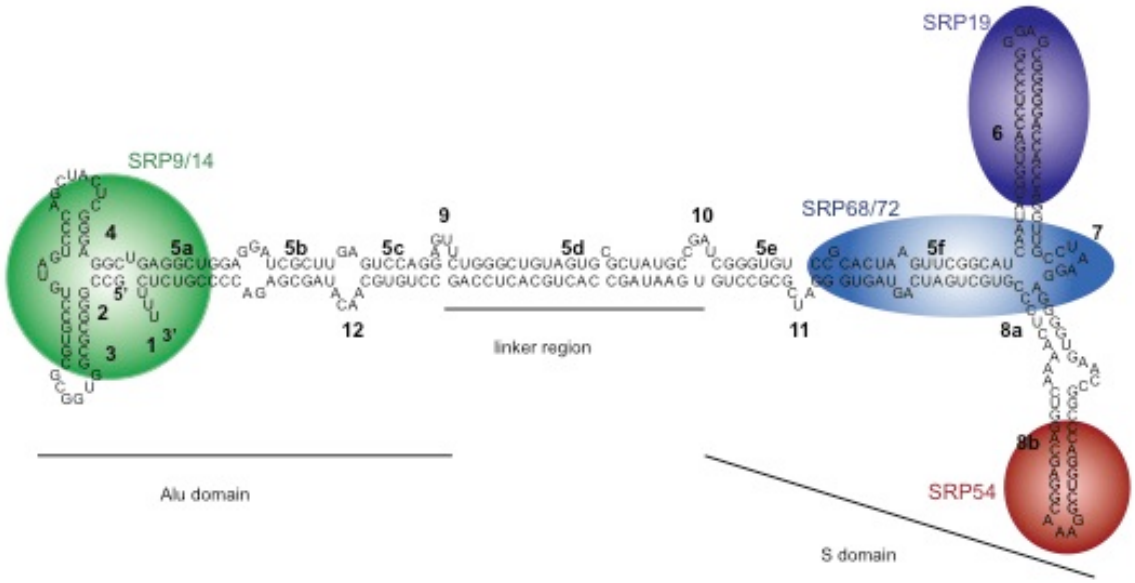

Figure 1-5. 2-dimensional schematic of 7SL RNA in complex with the SRP proteins. Bold numbers indicate the common nomenclature for the helices and loops of 7SL (343).

encode an N-terminal "signal sequence", characterized by a string of hydrophobic residues in an α-helical conformation, that is recognized by SRP54 (122). The *Alu* domain of 7SL RNA, with SRP9/14, subsequently binds the elongation factor-binding site of the ribosome, thus preventing elongation and arresting translation (122, 278). Binding of the signal sequence to SRP54 induces a conformational change in SRP54 and the S domain of 7SL, which causes the loading of SRP54 with GTP (38). The 7SL/SRP54 complex is thus ready to interact with the SR on the ER membrane. The ribosome/nascent polypeptide/SRP complex is subsequently translocated to the ER, although the details of this mechanism remain poorly understood (279). Once at the ER, the SRP docks with the SR – an interaction that requires the GTPase activity of SRP54 and is catalyzed by the S domain of 7SL – and the nascent polypeptide is threaded through the export channel in the ER membrane (245, 246). The SRP is released from the ribosome/SR complex upon GTP hydrolysis, and returns to the cytoplasm.

In addition it its role in the SRP, the *Alu* domain of 7SL is also the ancestor of the *Alu* retrotransposons that are found at a high frequency in both rodent and primate genomes (15, 262). Approximately 30% of the human genome is composed of *Alu* elements (17, 63), and a new *Alu* retrotransposon event is estimated to occur in 1 in every 20 births (64, 328). As retrotransposons, *Alu* elements require reverse transcription to mobilize. However, unlike long interspersed elements (LINEs), *Alu* elements do not encode their own RT, and must rely on an RT enzyme produced in *trans*. It is thought that *Alu* elements primarily employ the RT encoded by LINEs and other endogenous retrovirus-like elements (75). Given that the dramatic increase in the number of genomic *Alu* elements predates the divergence of the Euachontoglires lineage, which includes both

rodents and primates, and that this divergence occurred at least 85 mya (171), the association of 7SL with retroviruses or retrovirus-like elements can be assumed to be an ancient one.

Retroviral interactions with innate immunity and host restriction factors

The particular relationship of HIV with lymphocytes has resulted in the characterization of AIDS as a disease of the adaptive immune system. However, before the adaptive immune system is even triggered, HIV must avoid the innate immune system. Its ability to cause disease is a measure of how successfully it is able to do so.

Recognition by the innate immune system

To trigger an immune reaction, a pathogen must first be recognized as foreign. Mammalian cells employ pattern recognition receptors (PPRs), which recognize pathogen-associated molecular patterns (PAMPs) (213). PAMPs are motifs that are, by location or characteristics, unique to foreign molecules. The membrane-bound Toll-like receptors (TLRs) recognize either hydrophobic molecules such as glycoproteins (TLR1, 2, 4, 5, and 6, on the surface of the cell) or nucleic acids (TLR3, 7, 8, and 9, on endosomal compartments) (305). The cytoplasmic molecules RIG-I and MDA5 recognize cytoplasmic RNA; RIG-I recognizes short dsRNA and 5'-triphosphorylated RNA, while MDA5 recognizes long dsRNA and higher order RNA structures (332). The cytoplasmic molecules AIM2 and DAI similarly recognize cytoplasmic dsDNA (307). The recognition of a PAMP by a PPR triggers a signaling cascade that results in the

stimulation of interferon (IFN), NF-κB, and pro-inflammatory cytokines such as TNF-α and interleukin (IL)-6, all of which have antiviral effects (213).

HIV exhibits several PAMPs throughout its life cycle: its Env protein is heavily glycosylated, and at various points of reverse transcription it exhibits ssRNA, highly structured RNA, ssDNA, and dsDNA. However, HIV provokes little IFN response in both the acute and chronic stages of infection (91, 134, 157, 190). In part, this can be considered to be due to localization: TLR receptors for nucleic acids are on the interior of endosomal compartments, and HIV does not enter by endocytosis. The viral RNA and DNA products of reverse transcription are also thought to be shielded by capsid proteins during their journey through the cytosol: preventing their recognition by cytoplasmic PPRs such as MDA5 and DAI.

Although HIV avoids many of the PPRs early in infection, some recognition by the innate immune sensors inevitably occurs (214). RIG-I, TLR7, and TLR8 have been shown to recognize HIV (18, 117, 126). However, instead of curtailing infection, stimulation of these PPRs promotes the heightened (and destructive) immune response characteristic of acute and late-stage HIV infection (117, 181, 206). Moreover, the HIV transcriptional promoter contains NF-κB-responsive elements; the absence of NF-κB is one of the conditions that can drive latency in some cell types (12, 221). To prevent a strong IFN response but still maintain adequate levels of NF-κB, the viral proteins Vif and Vpr mediate the degradation of IRF3, which blocks the IFN pathway but leaves the NF-κB pathway intact (46, 80).

Host restriction factors

In addition to PPRs and other stimulatory molecules of the immune system, cells encode restriction factors that act directly against an infecting virus. Of these factors, four are of particular interest due to their efficacy against HIV: TRIM5α, tetherin, SAMHD1, and APOBEC3G (184).

TRIM5α is a cytosolic protein that interferes with the replication of HIV at an early stage post-entry (232, 281, 291). Although TRIM5α is constitutively expressed, it is also up-regulated in response to IFN (8, 44, 266). TRIM5α is saturatable by overexpression of CA. Accordingly, HIV's main defense against TRIM5α appears to be the genetic variability of CA (220, 232, 291). It is unclear exactly how TRIM5α interferes with viral replication, but there is some evidence that TRIM5α binds to CA and then targets the complex for degradation (76, 260). HIV is susceptible to the TRIM5α of Old World monkeys, but is not particularly susceptible to human TRIM5α – a classic example of the continuous "arms race" between pathogens and the immune systems of their hosts (282, 291). Surprisingly, human TRIM5α genes are exceptionally homogenous, a factor that might contribute to the ease of HIV infection in the human population (229, 269).

A recently discovered restriction factor is tetherin, an integral membrane glycoprotein (178). Previously, it had been noted that the viral protein Vpu was necessary for viral release in certain cell lines. Electron microscopy of ΔVpu virions in non-permissive cells revealed that virions were fully formed but "tethered" to the surface of the cells, often forming long chains of virions (123, 248). This tethering phenotype could be counteracted by mechanical shearing or proteolytic digestion, indicating that virions were fastened onto the cell by a protein rather than having failed to complete budding

(225). Genetic comparisons between permissive and non-permissive cell lines established that the protein responsible for this phenotype is Bst2, renamed tetherin (226). Vpu likely counteracts tetherin by targeting it for ubiquination and subsequent degradation via the endosome (111, 120, 202).

Another recently discovered restriction factor is SAMHD1. It had previously been recognized that HIV-1 replication is inefficient in dendritic cells and macrophages unless these cell types were co-infected with HIV-2 or sooty mangaby simian immunodeficiency virus (SIV_{SM}) or co-transfected with a plasmid expressing the HIV-2/SIV protein Vpx (161). By identifying the proteins that interacted with Vpx in non-permissive cell types, the restriction factor was identified as the protein SAMHD1 (136, 174). Vpx counteracts SAMHD1 by targeting it for proteasome-dependent degradation (2, 174). Recently, it was determined that SAMHD1 restricts retroviruses by depleting the cytoplasmic pool of deoxynucleoside triphosphates to below the level required to successfully complete reverse transcription (175). The cell-type specific restriction of HIV-1 by SAMHD1 is dependent on the expression of SAMHD1 – while dendritic cells and macrophages constitutively express SAMHD1, T cells do not, presumably because the rapid clonal expansion of T cells after activation requires a ready pool of deoxynucleosides (105, 175). Hence, HIV-1 replication is restricted in dendritic cells and macrophages but not in the CD4+ T cells that are its primary cell type.

The fourth highly studied restriction factor is APOBEC3G (A3G) (231, 274). A3G belongs to a family of cytosine deaminases unique to mammals, of which humans have seven (A-H) (145). A3 cytosine deaminases deaminate cytosines on ssDNA, yielding uracils (124, 179, 203, 337). Because uracils are not recognized as valid DNA

bases, the resulting heavily mutated DNA is often degraded by cellular factors (124, 330). If the proviral DNA escapes degradation and is successfully integrated into the host genome, the number of mutations can result in the expression of non-functional proteins. Despite the destructive effect that cytosine deamination has on proviral DNA, evidence suggests that the primary antiviral effect that A3G has on retroviral replication is through interfering with the action of RT during reverse transcription (25, 228, 312). Vif primarily counteracts A3G by targeting it for ubiquination and degradation, although it can also down-regulate A3G translation by binding to the 5' UTR of A3G mRNA (205, 209, 288, 334).

For A3G's antiviral effect, A3G must be present in producer cells, rather than target cells, despite the fact that the reverse transcription event that A3G acts upon occurs in target cells. This indicates that A3G is packaged into virions, and indeed, A3G is found at between 8 and 500 molecules per ΔVif virion, depending on the expression of A3G in the producer cell (34, 290).

The exact determinants for packaging of A3G remain controversial. In the cell, A3G forms either high molecular weight complexes associated with lipid rafts or low molecular weight complexes that reside in the cytosol; it is from the latter that active A3G is recruited (97, 194, 283). It has been well established that A3G packaging requires the NC domain of Gag, and evidence indicates that it is the basic regions of NC that are crucial for A3G packaging (37, 302, 336). A3G also likely requires multimerization to be packaged (37, 142). Both multimerization and interaction with NC appear to require RNA (26, 97, 142, 164, 336), but the debate continues regarding the identity of the necessary RNA. Some studies have found that A3G is not encapsidated by genome(-)

particles, indicating that gRNA is necessary for A3G packaging (163, 164). However, other studies have failed to replicate this result (311). Similarly, some studies have found that preventing 7SL from being encapsidated by overexpression of SRP19 prevents A3G from being encapsidated, indicating that it is 7SL that is responsible for mediating the packaging of A3G (309, 311). However, other studies failed to replicate these results (10, 163). A3G does interact with 7SL in the cell, although these interactions might be in the high MW complexes from which A3G is not recruited (54, 99).

Overview of Dissertation

This dissertation examines the packaging of 7SL RNA by HIV-1. 7SL is highly enriched in retroviral virions, including the virions of HIV-1. However, it remains unknown how 7SL is acquired by retroviruses, and what role, if any, 7SL plays in the retroviral life cycle.

In this dissertation, I sought to determine the mechanism of the packaging of 7SL RNA by HIV-1. To do this, I first examined the *trans*-acting packaging factors of 7SL, that is, the regions of the virus that are responsible for the packaging of 7SL. In Chapter 2, I discuss the evidence obtained from examining the packaging of 7SL by a panel of HIV-1 Gag mutants. We found that when the NC domain of Gag was ablated, only a fragment of 7SL corresponding to the S domain was retained in the viral particle. This fragment, called 7SLrem, was also retained when Gag consisted of only the CTD of CA and sp1.

Next, in Chapter 3, I examined the *cis*-acting packaging factors of 7SL, that is, the regions of the RNA that are responsible for its packaging. By creating truncations of 7SL that correspond to either the *Alu* domain or the S domain and examining their packaging phenotype, I determined that both the *Alu* and S domains of 7SL are capable of mediating packaging. However, only the *Alu* domain derivatives competed for packaging with endogenous 7SL, whereas the S domain derivatives were packaged in addition to endogenous 7SL. This suggests that there are two pathways by which 7SL can be acquired by HIV-1, an “additive” pathway and a “competitive” pathway. Furthermore,

the evidence that the *Alu* domain derivatives compete with endogenous 7SL for packaging implies that endogenous 7SL is packaged via interactions with its *Alu* domain.

In Chapter 4, I discuss some of the implications of my data, and propose further experiments through which the potential role of 7SL in HIV-1 assembly might be examined. I also provide further data on small RNA packaging by HIV-1, including evidence that Y RNAs, which are not usually packaged by HIV-1 virions, can be packaged by a specific Gag-only construct. Finally, I utilize our ability to manipulate the small RNA content of HIV-1 to induce HIV-1 to package a modified 7SL RNA containing an aptamer sequence, and speculate on potential uses of this capability.

Chapter Two

7SL is retained in minimal virus-like particles as an S domain fragment*

Abstract

HIV-1 is known to package several small cellular RNAs in addition to its genome. Previous work consistently demonstrates that the host structural RNA 7SL is abundant in HIV-1 virions, but has yielded conflicting results regarding whether or not 7SL is present in minimal assembly-competent virus-like particles (VLPs). Here we demonstrate that minimal HIV-1 VLPs retain 7SL RNA primarily as an endoribonucleolytic fragment, referred to as 7SL remnant (7SLrem). Nuclease mapping showed 7SLrem is a 111-nucleotide internal portion of 7SL, with 5' and 3' ends corresponding to unpaired loops in the 7SL two-dimensional structure. Analysis of VLPs comprised of differing subsets of Gag domains revealed that all NC-positive VLPs contained intact 7SL while the presence of 7SLrem correlated with the absence of the NC domain. Because 7SLrem, which maps to the 7SL S domain, was not detectable in infected cells, we propose a model whereby the species recruited to assembling VLPs is intact 7SL RNA, with 7SLrem produced by an

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endoribonuclease in the absence of NC. Since recruitment of 7SL RNA was a conserved feature of all tested minimal VLPs, our model further suggests that 7SL's recruitment is mediated, either directly or indirectly, through interactions with conserved features of all tested VLPs, such as the C-terminal domain of CA.

Introduction

Retroviruses such as HIV-1 exist as a collection of proteins and RNA; as such, they can be considered to be ribonucleoprotein complexes, assembled and released from the host cell under the direction of the viral genome. The viral structural protein Gag forms the core of the particle (reviewed in 100). Gag is expressed as a polyprotein consisting of six domains: matrix (MA), capsid (CA), spacer protein 1 (SP1), nucleocapsid (NC), spacer protein 2 (SP2), and p6. The domains of Gag are known to function in assembly as follows: MA binds to the plasma membrane via a co-translationally added myristate moiety; CA and SP1 promote Gag multimerization by protein-protein interactions; NC functions both in protein-protein interactions required for Gag multimerization and in genomic RNA recruitment; and p6 mediates virion release. Gag is the only viral protein required to form virus-like particles (VLPs) both *in vivo* and *in vitro*, but RNA is required for assembly under physiological conditions (58, 308). Furthermore, a minimal Gag – containing only the first twelve amino acids of MA, the C-terminus of CA plus SP1, and a leucine zipper motif replacing NC – efficiently produces VLPs in 293T cells (1).

The primary RNA present in viral particles is the genomic RNA (gRNA). Retroviruses package gRNA as a dimer, and HIV-1 gRNA packaging is mediated via

interactions between a region of gRNA known as the packaging signal (Ψ) and the NC domain of Gag (3, 67, 183). Although gRNA and NC are both dispensable for virus assembly, *in vitro* studies show some sort of RNA is required for assembly (218, 308). This implies that cellular RNAs are capable of playing some role in assembly, and indeed, up to 30% of the mass of RNA in retrovirus particles is of cellular origin (188). These include specific cellular RNAs which are present in concentrations that differ from those in the cell, implying that they are packaged specifically rather than randomly (21, 135, 188, 236, 237). The majority of cellular RNAs known to be packaged by retroviruses are small, non-coding pol III products that serve structural or enzymatic functions in the cell (236). The only cellular RNA present in retroviral particles whose viral function is known is the tRNA used to prime reverse transcription (166, 199).

One highly abundant cellular RNA, which is packaged by a broad range of retroviruses, is 7SL RNA: a component of the signal recognition particle (SRP). In the cell, the SRP serves to target nascent secretory or membrane polypeptides to the endoplasmic reticulum (reviewed in 66, 193). 7SL is composed of three domains: the *Alu* domain, the S domain, and the linker region, which separates the two (Fig. 2-1a). 7SL is the structural scaffold upon which the six proteins of the SRP bind. There is some evidence that a highly conserved region of 7SL RNA catalyzes the binding of SRP to its receptor SR, and that this RNA stimulates the GTPase activities of the SRP-SR complex without the presence of a GTPase-activating protein (245, 246). Previous work has determined that 7SL is present in HIV-1 particles at 6-8 copies

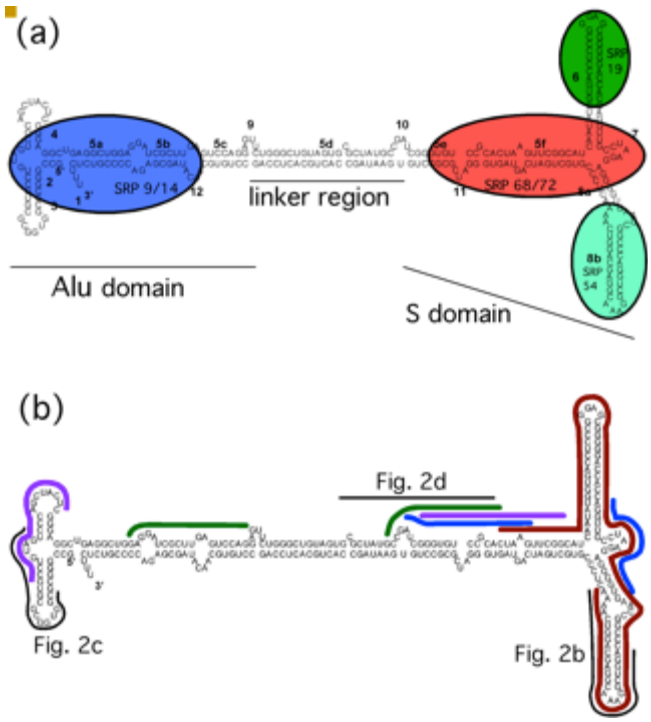

Figure 2-1. 7SL. (A) Schematic of human 7SL RNA, showing domains, nomenclature, and binding locations of signal recognition particle (SRP) proteins (193). (B) Locations of probes and RT-PCR primers used to determine the presence of 7SL in minimal virus-like particles. Thick lines represent RPA and RT-PCR studies performed previously: red, RPA from Onafuwa-Nuga *et al.* (237); blue, RT-PCR from Wang *et al.* (309, 310) and Bach *et al.* (10); purple, RT-PCR from Bach *et al.* (10); green, RT-PCR from Crist *et al.* (65). Thin lines represent probes used in this study, labeled to indicate which figure depicts the relevant experiment.

per genomic RNA, or approximately 14 copies per virion, and that encapsidated 7SL lacks its p54 protein component and thus is not packaged as part of the SRP (237).

A recent publication that concludes minimal virus-like particles lack detectable RNA seems to rule out the possibility that 7SL may be important to the virus (65). However, in the current study we demonstrate that a fragment of 7SL corresponding to the S domain is retained in minimal VLPs, at the same copy number as full-length 7SL in intact Gag forms of HIV-1. These and other data support a model in which 7SL RNA is recruited into assembling virions via portions of Gag that are retained in minimal VLPs, the nucleocapsid region of Gag subsequently protects the majority of 7SL from processing, and a host endonuclease interacts with HIV-1 at some point during assembly.

Materials and Methods

Cells and plasmids

293T cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Gemini). Cells were grown at 37°C under 5% CO_2. Virions containing processed Gag and Gag-Pol were produced by the plasmid pCMVΔR8.2, a Ψ-, *env*- proviral vector driven by the CMV promoter (224). VLPs containing intact Gag were produced by the plasmid pVR1012x/s Gag/h, which produces a protein identical to the Gag sequence of HXB2 (GenBank accession number K03455), but whose nucleotide sequence has been optimized for production in human cells (138). Minimal DNC VLPs, constructed by H. Gottlinger and colleagues, were described previously (1, 237). The pNL4-

3/Fyn(10) constructs were a generous gift from A. Ono (University of Michigan Medical School, Ann Arbor) (56, 238).

Transfection

Transfections of 293T cells were carried out on 100 mm plates. Virus and minimal ΔNC VLPs were produced by $CaPO_4$ transfection, as described previously (237). Fyn(10) virions were produced by PEI transfection. For transfection by PEI, plasmid DNA was mixed with 4 μg polyethelenimine (Polysciences) per μg DNA in 1 mL 150 mM NaCl by vortexing at medium speed for 10 s. After room temperature incubation for 15 minutes, the mixture was added dropwise to media on cells. The transfection mixture was not removed prior to virus harvesting.

Viral processing and RNA isolation

Tissue culture media was harvested at 24 h and 48 h post-transfection, pooled, and filtered through 0.2 μm filters. Virus was concentrated by centrifugation of virus-containing media through a 2 ml 20% sucrose cushion in PBS for 2 h at 4°C, 25,000 rpm using the Sorvall Surespin 630 rotor in a Sorvall Discovery 90 ultracentrifuge. Viral pellets were suspended in 0.5 ml Trizol reagent (Invitrogen), and RNA was isolated according to the manufacturer's instructions. Cellular RNA was obtained by harvesting cells 48 h after transfection by scraping cells into 2 ml of Trizol reagent per 100 mm plate, and RNA was isolated according to the manufacturer's instructions.

Northern blotting

Cellular and viral RNAs were separated by 8% polyacrylamide-8 M urea gel electrophoresis in 1X TBE buffer at 350 V for 2 h. The amount of viral RNA loaded in each lane was normalized to the volume of virus-containing media harvested, with each lane containing half of the RNA isolated from the virus produced by one transfected 100 mm plate over a 48 hour period. The amount of cellular RNA loaded per lane was 0.6 % of the total RNA isolated from a confluent 100 mm plate. RNA was transferred by electroblotting onto Zeta-probe GT nylon membranes (Bio-Rad) in 0.5X TBE buffer. After transfer, membranes were air dried and UV cross-linked (Stratalinker; Stratagene). Prehybridization was performed at 52°C for 1 h in 6X SSC-5X Denhardt's solution-0.5% (SDS)-0.025 M sodium phosphate (pH 6.5)-625 µg/ml of denatured salmon sperm DNA. Oligonucleotide probes were denatured by boiling at 100°C for 5 min, then radiolabled using ^{32}P-γATP (Perkin-Elmer) and T4 polynucleotide kinase (New England Biolabs). The *Alu* domain probe used in Fig. 2-2c was 5'-GACTACAGGCACGCGCCACCG-3'. The S domain probe used in Fig. 2-2b and Fig. 2-4 was 5'-TTTTGACCTGCTCCGTTTCCGACCT-3'. The linker region probe used in Fig. 2-2d was 5'-TGCGGACACCCGATCGGCATAGCGC-3'. Radiolabeled probes were added to the hybridization buffer and hybridization carried out at 52°C for 16 h. After hybridization, the blots were washed in 2X SSC-0.1% SDS at 50°C for 15 min, then 0.5X SSC-0.1% SDS at 50°C for 15 min, then 0.33X SSC-0.1% SDS at 50°C for 15 min. Damp blots were wrapped in saran wrap and exposed to phosphorimager screens. If re-probing with a new oligonucleotide probe was required, blots were stripped by washing three times in 0.1% SDS in ddH_2O at 80°C, then pre-hybridized

and probed as described above. Images were acquired by scanning with a Typhoon Trio Variable Mode Imager (Amersham Biosciences), and quantification was performed using the 1D gel imaging feature of ImageQuant TL, version 7.0 (GE Healthcare).

S1 nuclease mapping

A probe specific to the 3' half of 7SL was created by digesting the plasmid p7SL30.1 (344) with Bsu36I, which cleaves within the 7SL coding region between residues C170 and T171. The linearized plasmid was radiolabeled on both ends using ^{32}P-αdTTP (Perkin-Elmer) and Klenow DNA polymerase (New England Biolabs), then further digested with EcoRI, at a site downstream of 7SL. The resulting 160 base pair fragment was separated into single strands by boiling for five minutes, and purified by strand separation electrophoresis on a non-denaturing 5% polyacrylamide gel. S1 nuclease digestion to map an endoribonucleolytic fragment of 7SL, 7SLrem (for 7SLremnant), using this probe and cell and VLP-derived RNA samples, was carried out according to Green and Struhl (115). After S1 nuclease digestion, the samples were separated on a denaturing 8%-polyacrylamide sequencing gel and exposed to a phosphorimager screen.

Primer extension to map the 5' end of 7SLrem

An oligonucleotide probe specific to the S domain of 7SL, 5'-TTTTGACCTGCTCCGTTTCCGACCT-3', was radiolabeled using ^{32}P-γATP and T4 polynucleotide kinase and mixed with viral RNA. The probe-RNA mixture was

ethanol precipitated, resuspended in 20 ml of 1X MLV RT buffer (60 mM Tris (pH 8.3), 24 mM dithiothreitol, 0.7 mM $MnCl_2$, 75 mM NaCl, 0.06% NP-40, 6 μg/ml oligo(dT), 12 μg/ml poly(rA)), plus 0.5 mM dNTPs, and 1 ml Rnasin was added. After two minutes at 42°C, 200U of MLV RT (Promega) was added. The reaction was incubated at 42°C for 50 minutes and stopped by heating to 70°C for 15 minutes, then the samples were ethanol precipitated, run on a denaturing 8%-polyacrylamide sequencing gel and exposed to a phosphorimager screen.

Results

Minimal virus-like particles contain a fragment of 7SL. We previously reported that 7SL RNA is present in minimal virus-like particles and wild-type HIV-1 at indistinguishable levels (237). However, several subsequent studies, which also examined minimal VLPs for 7SL, observed vastly reduced or undetectable levels (10, 65, 302, 309, 310). In our initial work here, we sought to resolve these differences. Our earlier study used an RNase protection assay (RPA) to detect 7SL RNA, whereas 7SL was detected via RT-PCR in others' published work (Fig. 2-1b). The region protected by the RPA probe and the regions amplified by the RT-PCR primers were not equivalent. We reasoned that the discrepancies could be explained if only a fragment of 7SL – one that was recognized by the RPA probe but not encompassed by the RT-PCR primers – was present in minimal VLPs.

To test this notion, the RNA content of authentic HIV-1 Gag particles and minimal VLPs was examined by northern blotting (Fig. 2-2a). Virus-like particles

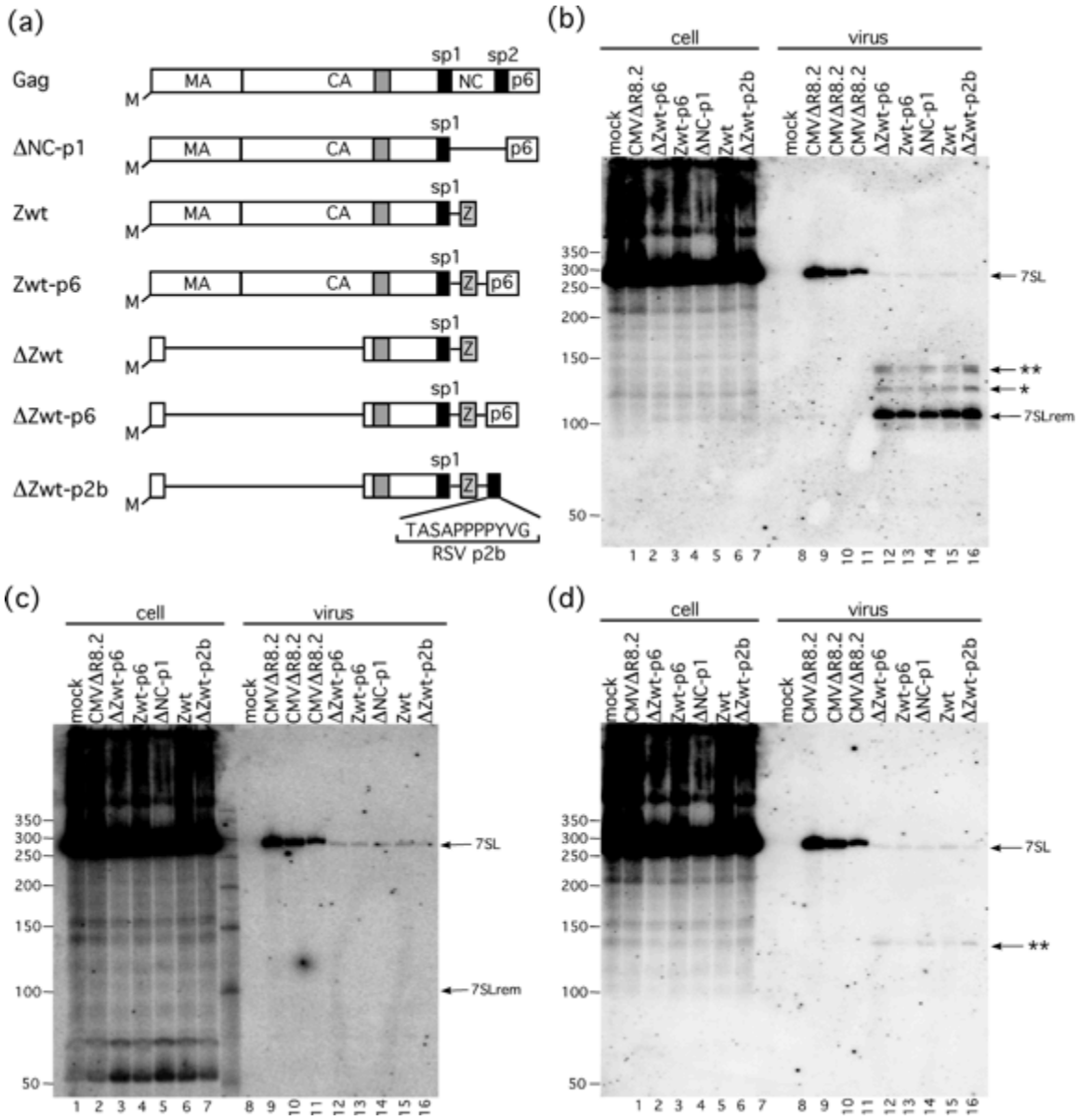

Figure 2-2. Retention of a fragment of 7SL in minimal virus-like particles. (A) Schematic representation of constructs here used to produce minimal virus-like particles. The dotted box within CA represents the major homology region. Lines represent deleted sequences. The myrisate moiety (M) is indicated. (Adapted from reference 1) (B, C, and D) Northern blots of RNA isolated from transfected cells and minimal VLPs. RNAs loaded in each lane are indicated at the top. Lanes 9 to 11 are 3-fold serial dilutions of virus from cells transfected with pCMVΔR8.2; RNAs in lanes 12-16 were from the same volume of transfected cell medium as lane 9. Probes used were specific to the S domain (B), *Alu* domain (C), and linker region (D), as indicated in Fig. 2-1. Full-length 7SL, 7SLrem, and the two remaining fragments (* and **) are indicated with arrows. RSV, Rous sarcoma virus.

were produced by transient transfection of VLP-expression plasmids into 293T cells. The constructs used were a series developed by Accola *et al.* (1) to address minimal assembly competent forms of HIV-1. As a positive control, intact Gag was provided by a Ψ- proviral construct, pCMVΔR8.2 (224). RNA was isolated from extracellular particles enriched by centrifugation through a sucrose cushion, and examined by northern blot. The northern blot was first probed with a radiolabeled oligo specific to the S domain of 7SL (Fig. 2-2b). The results showed that while both virions containing intact Gag and virus-like particles with minimal Gag packaged full length 7SL, the principal components of minimal VLPs were three fragments of 7SL: with the primary fragment migrating at approximately 100 nt, and two slightly larger fragments at lower abundance (indicated as **, *, and 7SLrem in Fig. 2-2b). The same blot was then stripped and re-probed with an oligo specific to the *Alu* domain of 7SL (Fig. 2-2c); this revealed only a small amount of full length 7SL in the minimal VLPs and no smaller fragments, indicating that the VLPs did not contain detectable fragments of 7SL which contain the *Alu* domain. Further probing of this blot, using a probe specific to the top strand of the 7SL linker region and a portion of the contiguous S domain, revealed only the largest of the three fragments (Fig. 2-2d), indicating that the increase in size comes from additional nucleotides on the 5' end of the fragments.

Quantifying probe hybridized to VLP RNA indicated that 2% of the total 7SL in the minimal VLPs was full length, the largest fragment was 9%, the second largest fragment was 8%, and the smallest fragment was 81% of the total of both full length

and fragmented 7SL. We have termed the smallest and most abundant fragment 7SL remnant, or 7SLrem.

Comparing the amount of 7SLrem in VLPs (lanes 12-16 in Fig. 2-2b) to the signal in an equal volume of virions containing authentic Gag (lane 9) suggested the molar amount of 7SL RNA and 7SL RNA derivatives in authentic and minimal Gag particles differed by less than 2-fold. It should be noted that whereas the changes introduced into some of these minimal VLP constructs were initially reported to result in large reductions in particle release (1), several other investigators have reported subsequently that under high level transfection in 293T cells, effects on virus release are not observed (reviewed in 95). Our laboratory's previous western blots of particles released from 293T cells transfected with the series of constructs used here have confirmed that particle release is not impaired detectably for these constructs, including ΔNC-p1 and Zwtp6 (237). These findings on virus release were confirmed in immunoprecipitation controls performed in support of the current work (data not shown).

No change in size or distribution of 7SLrem was detected in early-harvest virus collected from virus-producing cells one hour after fresh media change, as compared to virus harvested after 24 hours (data not shown), indicating that the apparent processing of 7SL to 7SLrem likely occurred at or before particle release.

The primary fragment of 7SL contained in minimal VLPs corresponds to 111 nt of the S domain. The RNA contents from virions containing intact Gag produced by the proviral vector pCMVΔR8.2, VLPs containing intact Gag produced by the Gag-

only expression plasmid pVR1012x/s Gag/h, or minimal VLPs produced by pZwt-p6, were compared further to determine the identity of 7SLrem. Specifically, the 3' and 5' ends of 7SL were mapped by S1 nuclease digestion and primer extension, respectively.

Figure 2-3a shows results of S1 mapping used to map the 3' end of 7SLrem. As evidenced by the 129 base fragment in lanes 1-6, all cell samples and both virions and VLPs that contain intact Gag contained only full-length 7SL and no detectable 7SLrem. This suggests that 7SLrem is not present as a discrete species within cells. Furthermore, the presence of intact 7SL RNA in VLPs that lack protease (Gag/h, lane 6) demonstrated that Gag processing is not required for incorporation of intact 7SL. The major band isolated from the minimal VLP Zwtp6 (lane 8) was 58 bases, which indicated that the 3' end of 7SLrem was C230.

Figure 2-3b shows results of the primer extension analysis used to map the 5' end of 7SLrem. Similar to Figure 2-3a, the major band in both species containing intact Gag, CMVΔR8.2 and Gag/h (lanes 1, 2), which was 216 bases, corresponds to full-length 7SL. The major band from the minimal VLP Zwtp6 (lane 5) is 98 bases, which indicates that the 5' end of 7SLrem was G120. Whether the numerous minor bands visualized for the Zwtp6 sample were due to pausing by RT on this highly structured RNA, alternate 7SL cleavage products, or other causes was not determined. The 5' ends of the two larger fragments were not determined with precision, but based on their mobilities on gels and hybridization patterns with additional probes (eg: Fig. 2-2d) were indicated to be approximately C111 and

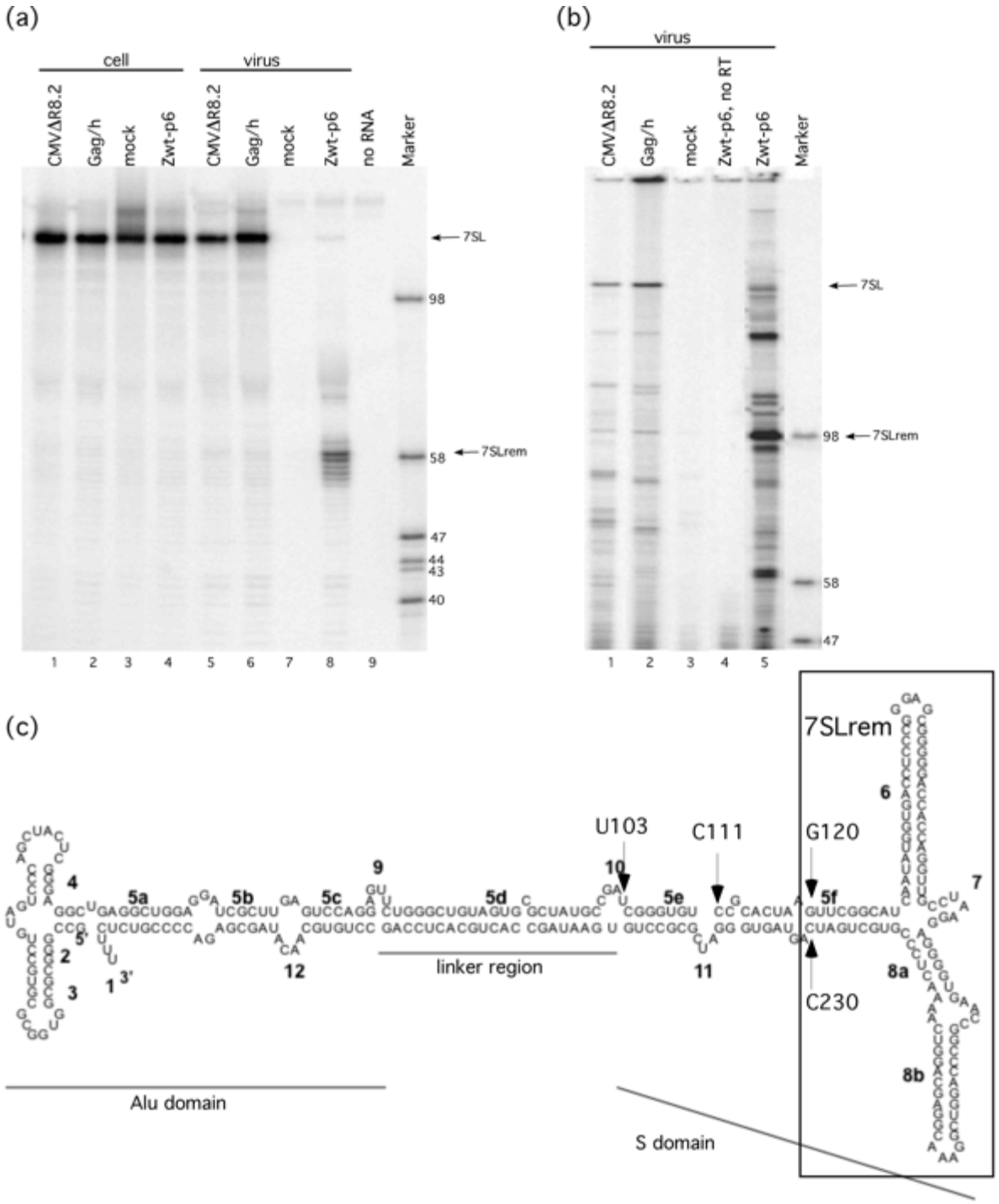

Figure 2-3. Identification of 7SLrem. (A) Sequencing gel of RNA fragments that have been hybridized to a probe specific to helix 8 of 7SL and digested with S1 nuclease in order to determine the 3' end of 7SLrem. (B) Sequencing gel of RNA fragments that have been hybridized to an oligonucleotide specific to helix 8 of 7SL and a primer extended by murine leukemia virus reverse transcriptase in order to determine the 5' end of 7SLrem. (C) A schematic of 7SL showing the 5' and 3' ends of 7SLrem determined as described above, as well as the inferred ends of the two larger 7SL fragments (indicated by arrows).

U103. Therefore 7SLrem can be mapped to 111 nt contained within the S domain of 7SL (Fig. 2-3c).

Full-length 7SL is present in ΔMA mutants containing NC, and 7SLrem is present in ΔMAΔNC mutants. The Gag mutants used in the above experiments retained the N-terminal 12 amino acids of MA to promote membrane localization. Thus, work with these mutants could not rule out interactions between 7SL and these residues of MA. Therefore, we examined the RNA content of viruses that did not contain any portion of MA. In the mutant Gag proteins used here, the first ten amino acids of Fyn kinase were fused onto the polyprotein N-terminus in order to recapitulate the membrane binding function of MA (Fig. 2-4a). Fyn(10) constructs recruit myristate moieties as MA does in the context of Gag, as well as two palmylate moieties, allowing binding to the plasma membrane, viral assembly, and release of viral particles (56).

The RNA content of Fyn(10) VLPs was examined by northern blot (Fig. 2-4b). In virions produced by Fyn(10) constructs that contained an intact NC domain (lanes 9, 10), full length 7SL was present at levels comparable to those found in VLPs containing authentic Gag expressed by the plasmid pVR1012x/s Gag/h (lane 8). In virions produced by Fyn(10) constructs which were missing the NC domain (lanes 11, 12), 7SLrem was present at levels comparable to those found in the minimal VLP ΔZwt (lane 13), regardless of whether or not the constructs contained the MA domain. Although these data were not normalized for viral particle production and therefore band intensities are not indicative of molar amounts of

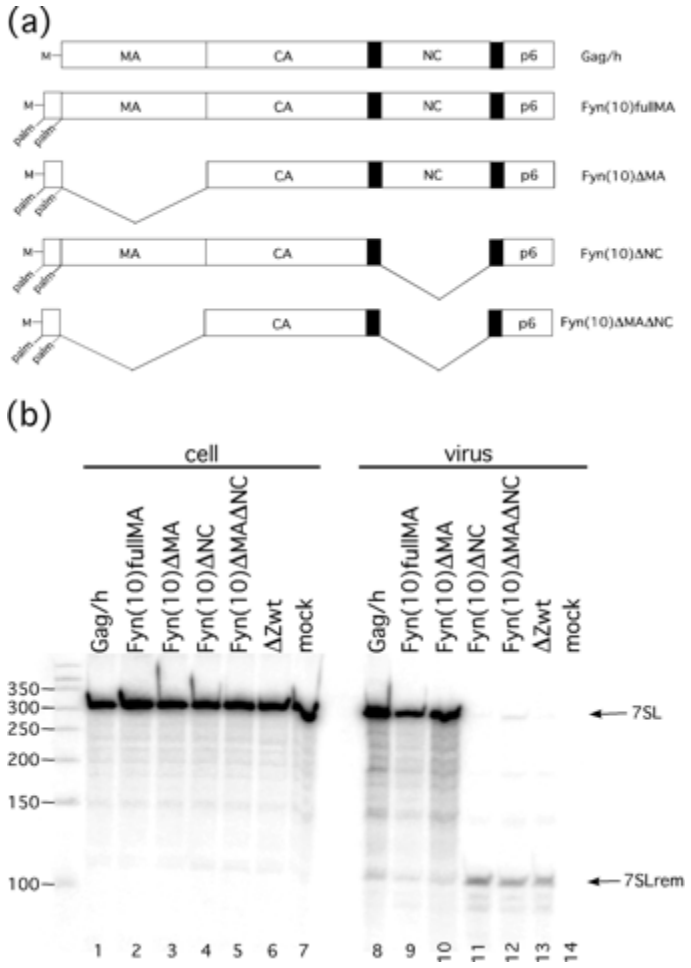

Figure 2-4. Packaging of 7SL in ΔMA virions. (A) Schematic of Gag derivatives with the addition of the N-terminal 10 amino acids of Fyn kinase. Myristate (M) and palmitate (palm) moieties are shown. (B) Northern blot of RNA from Fyn(10) constructions, as well as ΔZwt, a minimal VLP seen in Fig. 2-3, using a probe specific to the S domain.

7SL and 7SLrem, these data demonstrate that the packaging of neither full length nor processed 7SL was dependent upon any portion of matrix.

Discussion

We have examined the RNA content of minimal virus-like particles and determined that they contain three fragments of 7SL RNA. The primary fragment, termed 7SLrem, mapped to 111 nt within the S domain of 7SL, and the two secondary fragments were approximately 8 nt and 15 nt longer on the 5' end. 7SLrem was not detected in cells, nor does 7SL contain any regulatory sequences known to be capable of expressing only the 7SLrem region (87). Therefore, intact 7SL is probably the species recruited during assembly, and 7SLrem is likely processed from full-length 7SL while in association with viral components. This work was performed using virions produced by the highly-transfectable 293T cell type, which is known to release highly elevated levels of minimal VLPs (95). Due to the low yield of these VLPs from cells other than 293Ts, we have been unable to test the universality of the 7SLrem phenotype thus far.

Previous work assessing the RNA content of minimal VLPs yielded contradictory results, with nuclease protection revealing 7SL in minimal VLPs (237), but RT-PCR repeatedly failing to detect 7SL under similar circumstances (10, 65, 302, 309, 310). The data here resolve this conflict by demonstrating that while full-length 7SL is only present in small amounts in minimal VLPs, discrete 7SL fragments are observed in VLPs at the same molar levels as intact 7SL in authentic Gag particles. This explains why a probe previously used to detect 7SL by RPA (237),

which lies within the primary, and smallest, fragment of 7SL in VLPs, did detect this RNA, whereas the primers used for RT-PCR, which lie either completely or partially outside the retained sequence, detected little or no 7SL. In Crist *et al.*, the primers used lie completely outside of the 7SL fragments reported here, and no 7SL was detected in minimal VLPs (65). In Wang *et al.* (309, 310)primers lay outside all but the largest retained fragment. Our results (Fig. 2-2b) suggest this largest of the 7SL fragments, plus intact 7SL, comprised 11% of the total 7SL in the VLPs, which is in good agreement with the approximately ten-fold decrease reported in Wang *et al.* (309, 310). Bach *et al.* use two sets of primers, one completely outside of the retained fragments and one outside all but the largest retained fragment, and report 7SL levels in VLPs 2- or 12-fold reduced relative to those in WT virions (10). Crist *et al.* (65) not only failed to detect appreciable 7SL but also presented data showing no detectable RNA of any sort present in minimal VLPs. However, the reagent they used to monitor RNA, RiboGreen (Invitrogen), has been demonstrated to have low efficiency at quantifying RNAs around 100 bases or shorter, which is the approximate size of 7SLrem (152). The dependency of the packaging of the cytosine deaminase APOBEC3G on NC seen in Wang *et al.* (311) and Bach *et al.* (10) may also be explained by 7SLrem, and there is some evidence that packaging of APOBEC3G by HIV-1 may be dependent on 7SL (309, 311), although this remains controversial (163).

The ends of 7SLrem map to bulges in the secondary structure of the full length 7SL RNA where nucleotides remain unpaired. This, combined with the apparent precision with which the ends of 7SLrem are cleaved, implies processing

by a single-strand endonuclease. This endonuclease cannot be viral RNase H, as 7SLrem is found in Gag-only particles that lack this enzyme. Although no endonuclease has yet been identified in viral particles, the genomic RNA of retroviruses is often nicked when released from virions, implying encounters with an endonuclease (60, 235). Whether this endonuclease is the same as the endonuclease that appears to be acting on 7SL, and whether or not the endonuclease(s) in question are packaged into viral particles or associate only during assembly, remains to be determined.

It is interesting to note that the same processing of 7SL occurs whether the minimal VLP lacks only NC (as in ΔNCp1) or lacks the majority of Gag (ΔZwt). This indicates that nucleocapsid plays a role in protecting the full length RNA from processing. This protection does not require maturation to NCp7, as $Gag^{+}PR^{-}$ VLPs contained intact 7SL. It has been established that mutations to NC, specifically to the conserved CCHC motif of the zinc finger domains, severely reduce the packaging of genomic RNA and result in the production of non-infectious virus (reviewed in 300). When examined by electron microscopy, these viral particles exhibit a disordered, globular core rather than the compact, cone-shaped structure seen in WT particles (116). NC zinc finger mutants exhibit severe budding delays, and can initiate reverse transcription before the virion has fully released from the host cell (299). The proposed model for NC regulation of reverse transcription is that the CCHC motifs of NC zinc fingers promote the formation of a highly condensed core structure whereby reverse transcription is prevented until the dissolution of such a structure upon entry into the target cell (216). Such a model may explain 7SL processing in

ΔNC VLPs as well. Without the dense core structure produced by a fully intact NC domain, the endonuclease responsible for processing 7SL into 7SLrem may be able to function.

The processed forms of 7SL were retained in VLPs even when only a few domains of Gag were present. The VLP containing the least amount of Gag studied here (DZwt) contained only 8 amino acids of matrix, the C-terminal domain of capsid, and spacer protein 1. A role for the 8 amino acids of MA were ruled out using Fyn(10) constructs. Thus, the determinants of 7SL recruitment into HIV-1 must be either the CTD of CA and/or SP1, and/or the myristate moiety attached to the N-terminus of the polyprotein or resulting membrane localization. It remains to be shown whether 7SLrem interacts directly with Gag, or is retained in the virion through other means, such as in complex with another protein present in the viral particle.

Chapter Three

The *cis*-acting determinants of 7SL packaging by HIV-1*

Abstract

The host noncoding RNA 7SL is highly enriched in the virions of retroviruses. Here, we examined the regions of 7SL that mediate packaging by HIV-1. Both the *Alu* domain and the S domain were sufficient to mediate specific packaging when expressed separately as truncations of 7SL. However, while the *Alu* domain competed with endogenous 7SL for packaging in proportion to Gag, the S domain was packaged additively, implying that the *Alu* and S domains are packaged via separate mechanisms and that the *Alu* domain is packaged by the same mechanism as endogenous 7SL. Further truncations of the *Alu* domain or mutation of the *Alu* domain helix 5c region significantly reduced packaging efficiency, implicating helix 5c as critical for packaging, reinforcing the finding that 7SL packaging is highly selective, and confirming that 7SL is not passively acquired. Surprisingly, when the *Alu* domain was mutated so that it no longer contained a binding site for the SRP protein heterodimer SRP9/14, it was no longer packaged in a competitive manner, but instead was packaged additively with endogenous 7SL. These data support a

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model in which 7SL RNA is packaged via interactions between Gag and a 7SL RNA structure that exists transiently at a discrete stage of SRP biogenesis. Our data further indicate that a secondary "additive" pathway exists that can result in the packaging of certain 7SL derivatives in molar excess to endogenously packaged 7SL.

Introduction

Retroviruses, like all RNA viruses, are ribonucleoprotein (RNP) complexes. The primary protein component of retroviral particles is the structural polypeptide Gag, while the primary RNA component is the viral genomic RNA (gRNA). *In vitro*, nucleic acids or similar molecules are necessary to trigger the assembly of soluble Gag proteins into virus-like particles (40, 41). These RNA/protein interactions, some of which remain poorly understood, are critical during retroviral assembly.

The nucleocapsid (NC) domain is the primary region of Gag involved in interactions with RNA. Although it contributes to many viral functions, in simple terms NC contains RNA-binding motifs that either interact specifically with gRNA (the "zinc finger" motifs) or engage in non-specific interactions with nucleic acids (the basic regions). Mutations in the latter dramatically reduce intact particle release, while mutations in the former do not (58, 308). Moreover, Gag can form VLPs in the absence of viral gRNA (3, 263). Gag molecules from which NC has been deleted entirely form VLPs less efficiently than wild-type Gag; however, this phenotype can be rescued if NC is replaced with a sequence capable of forming protein-protein interactions, such as a leucine zipper motif (1, 65, 340). Taken together, these data have led to a model in which RNA, but not necessarily the viral

genome, promotes the multimerization of Gag through interactions with the NC domain during retroviral assembly.

Although the primary RNA component of retroviral particles is gRNA, retroviruses also package cellular RNAs, and as much as 30% of the RNA in a retroviral particle is host-derived (21, 24, 135, 188, 263). These RNAs are primarily small, non-coding, highly structured RNA polymerase III (Pol III) products, and some are packaged in molar excess over the genomic RNA (236, 237). One such Pol III product is the 301 nt 7SL RNA, which has been recognized as a component of retroviral particles since the 1970s (24). Those retroviruses for which RNA content has been studied in detail display replicable patterns of host RNA packaging, implying non-random acquisition of these RNAs. For example, in murine leukemia virus (MLV), the RNAs mY1, mY3, 7SL, and the murine 7SL derivative B1 are highly enriched in the virion (104, 236). Similarly, in human immunodeficiency virus type 1 (HIV-1), 7SL is highly enriched (77, 237). However, the human 7SL derivative scAlu RNA and the hY1 and hY3 RNAs are not present in appreciable amounts in HIV-1 (237, 302, 309). With the exception of the tRNAs that retroviruses use to prime reverse transcription (166, 199), any functions of these packaged host RNAs in viral replication remain unknown.

7SL RNA is well-packaged by many retroviruses (24, 89). It is found at 3-4-fold molar excess over a monomer of MLV genomic RNA and at 6-7-fold molar excess over the genomic RNA of HIV (236, 237). 7SL has been implicated in the packaging of the cytidine deaminase APOBEC3G by HIV-1 (309-311, 338), although this finding remains controversial (10, 163, 289). Evidence for interactions between

7SL derivatives and retrotransposition machinery extends beyond exogenous retroviruses, as the highly repetitive *Alu* sequences found in primate and rodent genomes are derived from the *Alu* domain of 7SL (17, 262). *Alu* elements are retrotransposons, mobilized by retrovirus-like elements present in the genome. The origin of genomic *Alu* elements can be traced back at least to the origin of the Euarchontoglires lineage (171), which includes both primates and rodents and which diverged ~85 mya. The association between 7SL and retroviruses can therefore be considered an ancient one.

The normal function of 7SL in the cell is as the RNA scaffold of the signal recognition particle (SRP), a ribonucleoprotein complex that is responsible for targeting nascent secretory and transmembrane polypeptides to the endoplasmic reticulum (ER) (for reviews see (66, 193)). 7SL RNA is composed of three domains (Fig. 3-1): (i) the *Alu* domain, which binds the SRP proteins SRP9 and SRP14 and is responsible for arresting translation of the nascent polypeptide upon binding to the ribosome complex (122, 140, 292); (ii) the S domain, which binds the SRP proteins SRP 19, SRP 54, SRP 68, and SRP 72 and is responsible for targeting the arrested ribosome to the ER (113, 208, 277, 279); and (iii) the linker region, which separates the *Alu* and S domains. 7SL is also capable of acting as an enzyme, in the sense that a highly conserved region of the S domain, helix 8, is responsible for catalyzing the docking between SRP and the SRP receptor (245, 246). The fact that RNA (but not genomic RNA) is necessary for retroviral assembly, paired with the structural role of 7SL in the cell and its high abundance and selective packaging in at least most kinds

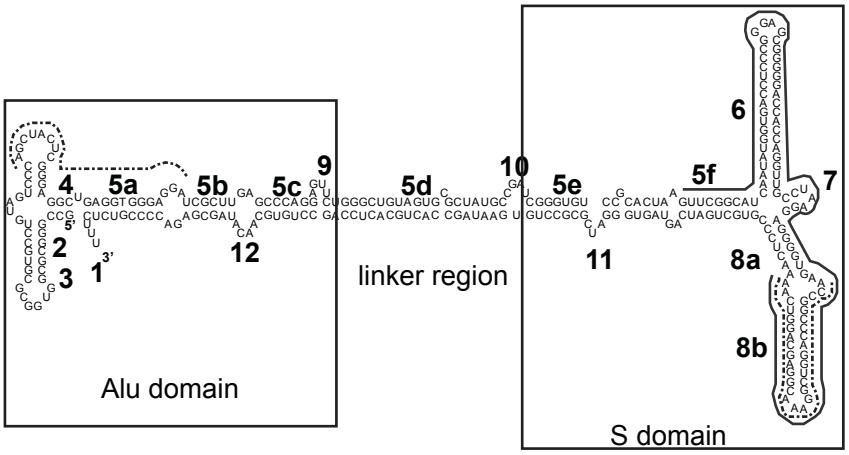

Figure 3-1. Secondary structure of human 7SL RNA, allele B. *Alu* and S domains are boxed. Bold numbers indicate previously established nomenclature for 7SL helices and loops (343). These designations are preserved throughout this paper to indicate the origins of specific 7SL segments. The dashed lines represent the probes used for northern blotting, and the solid line represents the probe used in the RNase protection assays.

of retroviral particles, appears consistent with the possibility that 7SL may play a structural role in retroviral assembly.

Previously, we identified the *trans*-acting determinants of 7SL packaging (162). We demonstrated that even minimal assembly-competent forms of HIV Gag that retain only the C-terminal domain (CTD) of capsid (CA) and spacer protein 1 (sp1) package 7SL RNA as efficiently as intact Gag, but that only a fragment of 7SL was observed in particles that lacked NC. This finding indicates that an interaction with CA/sp1 is sufficient for 7SL acquisition but that the *Alu* domain of 7SL is protected from endonucleolytic processing when NC is present (162). Here, we generated 7SL mutants and tested their packaging to define the *cis*-acting sequences that mediate specific 7SL packaging by HIV-1.

Materials and Methods

Plasmids

Most 7SL-derivative expression plasmids were made by overlap extension PCR templated by the plasmid p7SL30.1 (344), which contains human 7SL allele B with its endogenous promoter, and inserting the PCR products into the backbone of p7SL30.1 in place of the WT 7SL coding region. 7SL derivatives expressed by the U6 promoter were made synthetically by filling out overlapping 60 nt primers using Taq DNA polymerase. These products were inserted into the multiple cloning site of the plasmid pBlueScript KS+. HIV-1 virions were produced by transfecting 293T or ET cells (250) with the plasmid pHIVpuro, a ΔEnv, ΔVif, ΔVpr version of HIV-1 with the *env* coding region replaced by a gene coding for puromycin resistance (176).

Templates for riboprobes that recognize HIV-1 genomic RNA and 7SL (pSRK1520-1 and pBRU-7SL, respectively) were previously described (192, 237).

Cells and transfections

Virus was produced in 293T or ET cells (a 293T derivative that constitutively expresses ecotropic envelope (250)), which were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gemini), at 37°C under 5% CO_2. Target cells were D17/pJET cells (canine cells that constitutively express the ecotropic receptor (233)), which were cultured and maintained in DMEM supplemented with 10% bovine serum (CS; Gemini). Transfection was carried out on 100 mm plates using polyethelenimine (PEI; Polysciences Inc), as described previously (162). For each co-transfection of a plasmid that expressed a 7SL derivative with pHIVpuro (the plasmid that expressed the HIV virus-like particles), 8 μg of each plasmid was used. Plasmid DNA was replaced by purified herring sperm DNA (Roche) in the mock samples and the samples that expressed HIV without exogenous 7SL so that the total amount of DNA and PEI used remained constant. The media + transfection mixture was removed and replaced with 10 mL of fresh media 24 h after transfection.

Virus processing and RNA isolation

Tissue culture media was harvested 48 h after transfection and filtered through 0.22 μm filters. Virus was quantified by a reverse transcriptase (RT) assay as described

previously (297). Virus was concentrated from 10 mL media by centrifugation through a 2 mL 20% sucrose cushion in phosphate-buffered saline (PBS) for 2 h at 4°C and 25,000 rpm using a Sorvall Surespin 630 rotor in a Sorvall Discovery 90 ultracentrifuge. Pellets were resuspended in 500 µl Trizol (Invitrogen), and RNA was isolated according to the manufacturer's instructions. Cells were harvested 48 h after transfection by scraping cells into 2 mL Trizol per 100 mm plate, and RNA was isolated according to the manufacturer's instructions.

Northern blotting

Northern blotting was carried out as described previously (162). Loading was normalized so that RNA from equivalent amounts of virus was loaded in each lane. For cellular RNA, 0.6% of the total RNA isolated from a confluent 100 mm plate was loaded in each lane. The locations recognized by the oligonucleotide probes used are indicated with dashed lines on Fig. 3-1 and were as follows: to detect the *Alu* domain (Fig. 3-2b, Fig. 3-3b, Fig. 3-5a), 5'-ATCCTCCAGCCTCAGCCTCCCGAGTAGCTG -3'; to detect the S domain (Fig. 3-4b), 5'-TTTTGACCTGCTCCGTTTCCGACCT-3'. Images were acquired by scanning with a Typhoon Trio Variable Mode Imager, and quantification was performed using the 1D gel imaging feature of ImageQuant TL, version 7.0.

RNase Protection Assay

RNase protection assays (RPAs) were performed as previously described (236). The riboprobes used recognized either a portion of the HIV-1 *gag* gene (CA/sp/NC) or the S domain of 7SL from C116 to C217 (indicated as a solid line on Fig. 3-1).

Titer

HIVpuro virus for infection was produced by transfection of ET cells as above. Media was harvested after 48 h and filtered through 0.22 μm filters. 100 mm plates of D17/pJET cells were infected with 2 mL DMEM + 10% CS, 5 μg/mL polybrene (Sigma), and serial dilutions of HIV-containing media. Two hours later, the media was discarded and 10 mL of fresh media was added to each plate. After 2 days incubation, media was replaced with DMEM + 10% CS + 2 μg/mL puromycin (Sigma), which was changed every two days until colonies were visible, 12-14 days after infection. Colonies were stained with trypan blue and counted.

Statistical analysis

Statistical significance was analyzed by performing two-tailed, unpaired Student's *t* test on pairs of data, using the statistical functions of Microsoft Excel. Differences were considered significant if $p < 0.05$.

Results

An isolated *Alu* domain competes with endogenous 7SL for packaging. To determine the minimal region of 7SL capable of mediating specific packaging,

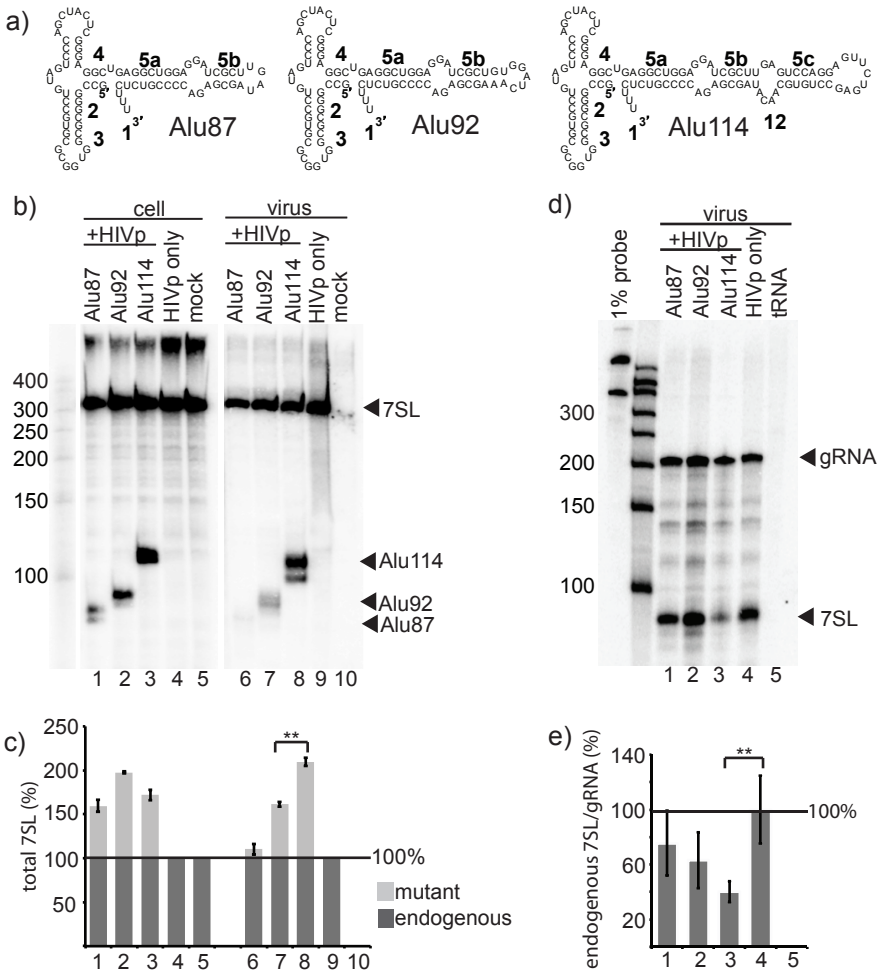

Figure 3-2. The *Alu* domain is sufficient for packaging. A) Schematics of the *Alu* domain derivatives used. Alu114 corresponded to the entire *Alu* domain, and Alu92 and Alu87 were successively shorter truncations. B) Northern blot of RNA from co-transfected cells and HIVp virus, showing the expression and packaging of the *Alu* domain derivatives. The probe used was against the *Alu* domain of 7SL. The multiple bands observed for the *Alu* derivatives likely reflect alternate 3' end modifications (52); virion encapsidation of subsets of Pol III processing products has been described previously (104). C) Quantification of mutant (light gray) and endogenous (dark gray) 7SL RNA from northern blots. Column numbers correspond to the lane numbers in panel B. The mean amount of total 7SL is shown; endogenous 7SL was set to 100% for cells and virus. D) RPA of RNA from HIVp virus. Riboprobes used were against the S domain of 7SL and HIV genomic RNA (gRNA). E) Quantification of RPAs of HIVp virus RNA. Column numbers correspond to the lane numbers in panel D; gRNA = genomic RNA. The mean amount of endogenous 7SL RNA per gRNA is shown; the 7SL/gRNA value of the HIVp-only sample was set at 100%. Error bars, ±S.D.; ** = $p<0.01$; n=3 independent experiments.

truncations of 7SL were created that corresponded to successively shorter regions of the *Alu* domain. As diagrammed in Fig. 3-2a, Alu114 contained an internal deletion in 7SL from G82 to C268, thus yielding a 114 nt RNA that corresponded to the entire *Alu* domain, while Alu92 and Alu87 contained larger deletions and thus encoded smaller RNAs. Alu114 corresponds to the same region of 7SL that is the ancestor of the murine 7SL derivative B1, which is packaged by MLV (236), and of the human 7SL derivative scAlu. The *Alu* domain derivatives were co-transfected with HIVpuro ((176), a plasmid that expresses HIV virus-like particles that contain all viral proteins except Env, Vif, and Vpr and will henceforth be referred to as HIVp), and the RNA content of co-transfected cells and HIVp virions produced from these cells was examined by northern blotting (Fig. 3-2b). Although expression of all three *Alu* domain truncations was readily detectable in co-transfected cells (Fig. 3-2b, lanes 1-3), only Alu114 was packaged in high quantities in virions (lane 8), with Alu92 and Alu87 packaged less well (lanes 6 and 7). Quantification of these blots revealed that Alu114 comprised approximately 50% of the total 7SL (endogenous 7SL and Alu114) in HIVp virions from co-transfected cells (Fig. 3-2c), whereas Alu92 comprised approximately 35% and Alu87 comprised approximately 10% of the total 7SL. The amount of Alu114 and Alu92 that was packaged by HIVp virions was significantly different ($p<0.01$). Another way of addressing packaging selectivity is to determine how enriched specific RNAs are, by comparing their levels in virions to their levels in cells. If the enrichment factor of endogenous 7SL was set to 1, Alu114 was found to be enriched 1.5-fold in the virus over its concentration in the cell.

The amount of endogenous 7SL per virion was subsequently quantified by performing a ribonuclease protection assay (RPA) using two riboprobes: one to the 7SL S domain that recognized endogenous 7SL, but not Alu114, and one that recognized viral genomic RNA (Fig. 3-2d). Reverse transcription (RT) assays were performed on the viral samples to quantify viral proteins, and the amount of genomic RNA per sample was found to be proportional to RT for each sample. Thus, detection of genomic RNA allowed for normalization to the number of virions. Comparing the endogenous 7SL signal for the HIVp-only virus (lane 4) to that for the Alu114-containing virus (lane 3) showed that the ratio of endogenous 7SL to gRNA in virions was approximately two-fold lower in HIVp virions produced by co-transfected cells in which ~40% of the total 7SL-type RNA was Alu114 than it was in virions produced in the absence of Alu114 (Fig. 3-2e). This difference in endogenous 7SL packaging was statistically significant ($p<0.01$). Similarly, the amount of endogenous 7SL was reduced approximately 35% in HIVp virions containing Alu92, and by approximately 14% in HIVp virions containing Alu87. Paired with Fig. 3-2c data, these findings indicated that the total amount of 7SL-type RNAs (7SL and Alu114, Alu92, or Alu87) in virions remained constant, implying that the Alu domain derivatives competed for packaging with endogenous 7SL. Based on previously determined endogenous 7SL levels packaging levels (12-14 copies), these data indicate that under the conditions here, 6-7 molecules of Alu114 replaced the corresponding number of endogenous 7SL molecules per viral particle, or approximately 4 molecules of Alu92 or 1 molecule of Alu87 was packaged per virion when these later derivatives were co-expressed.

Helix 5c, but not binding to SRP9/14, is required for packaging of the *Alu* domain. Next, the difference between the well-packaged Alu114 and the poorly packaged truncations was dissected to define the packaging signals in the *Alu* domain. The primary difference between these RNAs was the presence of helix 5c (H5c) in Alu114 (Fig. 3-1 and Fig. 3-3a). To investigate whether the length, secondary structure, flexibility, or sequence of H5c accounted for the increased packaging of Alu114, H5c of Alu114 was mutated to produce Alu114 helix(+) and Alu114 helix(-) (Fig. 3-3a). In Alu114 helix(+), the sequence of H5c was mutated in a way predicted to maintain the H5c helix-loop conformation; in Alu114 helix(-), both the sequence and the predicted secondary structure of H5c were altered.

To assess the effects of H5c modification on RNA packaging, northern blotting was performed on RNA from cells co-transfected with HIVp and the *Alu* domain derivatives, and on RNA from virions produced from these cells (Fig. 3-3b). Although expression of both constructs was detectable in cells (lanes 3 and 4), neither *Alu* derivative was present in the virus above the threshold of detection. By quantifying the background signal and the signal of packaged Alu114, we determined that both helix(+) and helix(-) RNAs were packaged at least 6-fold less efficiently than Alu114 (compare lane 7 to lanes 9 and 10). Quantification of these northern data is presented in Fig. 3-3c. These results suggest that the specific sequence of H5c, rather than its length or basic features of its secondary structure, was crucial for packaging.

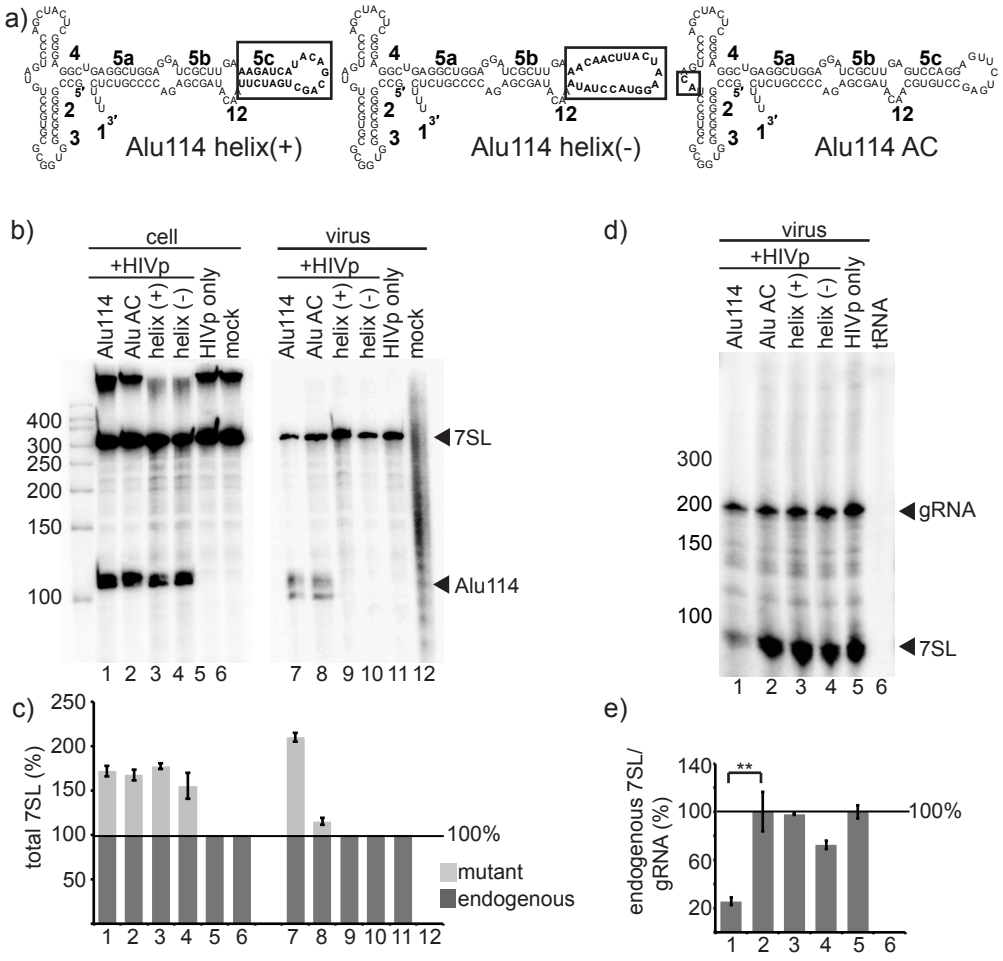

Figure 3-3. Disruption of helix 5c, but not interference with SRP9/14 binding, abrogates packaging of the *Alu* domain. A) Schematics of mutants of the *Alu* domain derivative Alu114. The mutated regions are boxed. B) Northern blot of RNA from transfected cells and HIVp virus, showing the expression and packaging of Alu114 and the *Alu* domain mutants. The probe used recognized the *Alu* domain. C) Quantification of mutant (light gray) and endogenous (dark gray) 7SL RNA from northern blots. Column numbers correspond to the lane numbers in panel B. The mean amount of total 7SL is shown; endogenous 7SL was set to 100% for cells and virus. E) Quantification of RNA from HIVp virus based on RPAs like than in panel D. Column numbers correspond to the lane numbers in panel D. The mean amount of endogenous 7SL RNA per genomic RNA is shown; the value of endogenous 7SL/gRNA of the HIVp-only sample was set at 100%. Error bars, ±S.D.; ** = $p<0.01$; n=3 independent experiments.

To further address *Alu* packaging determinants, the roles of this RNA's protein binding partners were assessed. During SRP biogenesis, the first proteins that bind nascent 7SL are the protein heterodimer SRP9/14, which forms a complex with the *Alu* domain (52). In initial work not shown, the SRP9/14 protein content of virions was examined by western blotting to determine whether SRP9/14 was packaged by HIVp. Although the results indicated that the SRP9/14 in virions was at least 8-fold de-enriched compared to cells when normalized to 7SL content, this sensitivity was not high enough to conclusively exclude SRP9/14 packaging in virions because SRP9/14 exists in 20-fold excess over 7SL in the cell (30).

Therefore, the possible role of SRP9/14 binding was examined indirectly, by creating a derivative of Alu114 in which the binding site for SRP9/14 was abolished by changing the nucleotides G24 and U25 to A and C, respectively (Fig. 3-3a, henceforth referred to as Alu AC). In RNA/protein co-crystals, G24 and U25 are the only nucleotides of 7SL that are specifically recognized by SRP9/14 (317), and mutations to G24 and U25 reduce SRP9/14 binding up to 50-fold *in vitro* (47, 86). To assess the influence of an intact SRP9/14 binding site on the packaging of Alu114, cells were co-transfected with HIVp and the Alu AC expression construct, and northern blotting was performed on the RNA content of these cells and the virions produced by them (Fig. 3-3b). Similar to observations with the unmutated Alu114 derivative (lanes 1 and 7), the Alu AC mutant was detectable in both cells (lane 2) and virus (lane 8). These results are quantified in Fig. 3-3c. The relatively efficient packaging of Alu AC suggested that the binding of SRP9/14 was not required for the packaging of the *Alu* domain.

We subsequently quantified the amount of endogenous 7SL packaged in the presence of the Alu AC mutant by RPA (Fig. 3-3d). Unlike the significant decrease in endogenous 7SL observed in virions containing Alu114 (lane 1), the endogenous 7SL signal in virions containing the Alu AC mutant remained unchanged compared to gRNA (compare lane 2 to lane 5). Quantification of the RPA confirmed that the endogenous 7SL to gRNA ratio decreased in Alu114-containing virions, but not in Alu AC-containing virions (Fig. 3-3e). The difference in the packaging of endogenous 7SL between the Alu114-containing virions and the Alu AC-containing virions was statistically significant ($p<0.01$). These data indicate that while binding to SRP9/14 was not required for packaging of the *Alu* domain, the mechanism by which the *Alu* domain was packaged was no longer competitive with endogenous 7SL when its interaction with SRP9/14 was ablated.

S domain-only RNAs are packaged in addition to endogenous 7SL. Truncations of 7SL corresponding to isolated S domains were also created to determine the influence of the S domain on 7SL packaging. The S domain is the most highly conserved portion of SRP RNAs, and orthologs of the S domain RNA are found in all kingdoms of life (66). Because the 7SL promoter contains intragenic promoter elements within the *Alu* domain (87), an isolated S domain could not be expressed under the wild-type 7SL promoter. Therefore, the S domain derivatives S154 and S114 were expressed using the U6 promoter, a Pol III type III promoter that contains only extragenic promoter elements (244) (Fig. 3-4a). S154 corresponded to the entirety of the S domain, while S114 corresponded to the 111 nt fragment of the

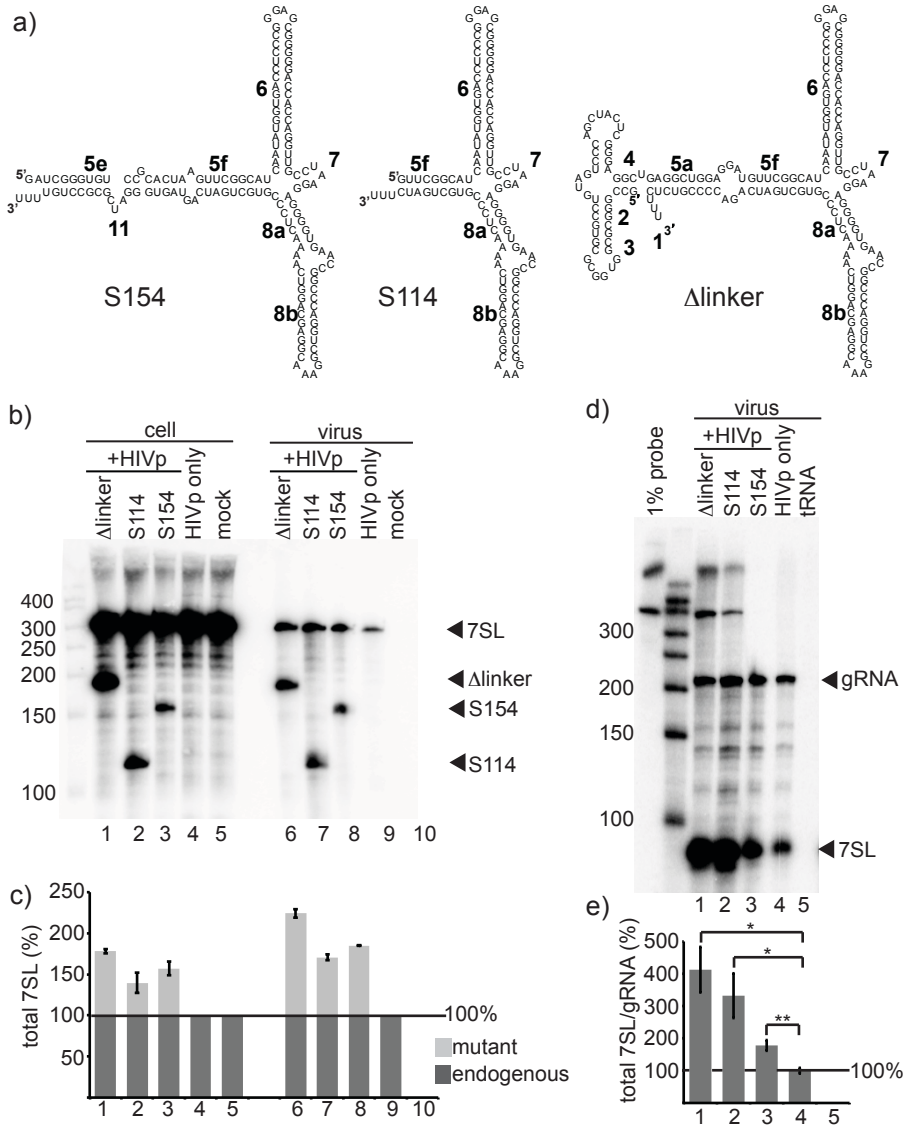

Figure 3-4. The S domain mediates additive packaging. A) Schematics of the S domain derivatives used. B) Northern blot of RNA from transfected cells and HIVp virus, showing the expression and packaging of the S domain derivatives. The probe used recognizes the S domain. C) Quantification of mutant (light gray) and endogenous (dark gray) 7SL RNA from northern blots. Column numbers correspond to the lane numbers in panel B. The mean amount of total 7SL is shown; endogenous 7SL was set to 100% for cells and virus. D) RPA of RNA from HIVp virus. E) Quantification of RPAs of RNA from HIVp virus. Column numbers correspond to the lane numbers in panel D. The mean amount of endogenous 7SL RNA per genomic RNA is shown; the value of 7SL/gRNA of the HIVp-only sample was set at 100%. Error bars, ±S.D.; * = $p<0.05$; ** = $p<0.01$; n=3 independent experiments.

S domain that is retained in minimal VLPs (162). To exclude the possibility that any phenotypes of the S domain RNAs resulted from use of the heterologous U6 promoter, a third S domain derivative, termed Δlinker, was constructed in which the S domain was expressed by the native 7SL promoter. The Δlinker mutant contained two deletions in 7SL, from C61 to A119 and from A231 to G286, which removed the7SL linker region RNA stem and parts of the *Alu* and S domains (Fig. 3-4a). The left-hand 75 nt of Δlinker, corresponding to part of the *Alu* domain (helices 2, 3, 4, and

5a), contained the intragenic promoter elements. However, as determined in Fig. 3-2 and data not shown, this portion of 7SL was incapable of mediating packaging (Fig. 3-2). The right-hand 111 nt of Δlinker corresponded to the S114 derivative described above (helices 5f, 6, 7, and 8).

The RNA content of virions produced by cells co-transfected with expression constructs for these S domain derivatives and HIVp was examined by northern blotting (Fig. 3-4b). S154 and S114 were detected in both cells (lanes 2 and 3) and HIVp virus (lanes 7 and 8). The mutant Δlinker was also packaged by HIVp (Fig. 3-4b, lane 6), confirming that the S domain was capable of mediating packaging regardless of its promoter. These results are quantified in Fig. 3-4c. If the enrichment factor of endogenous 7SL was set to 1, S114 was found to be enriched 1.8-fold in the virus over its concentration in the cell, S154 was found to be enriched 1.5-fold in the virus over its concentration in the cell, and Δlinker was found to be enriched 1.6-fold in the virus over its concentration in the cell. We also quantified the total 7SL RNA content (S domain derivatives and endogenous 7SL) compared to

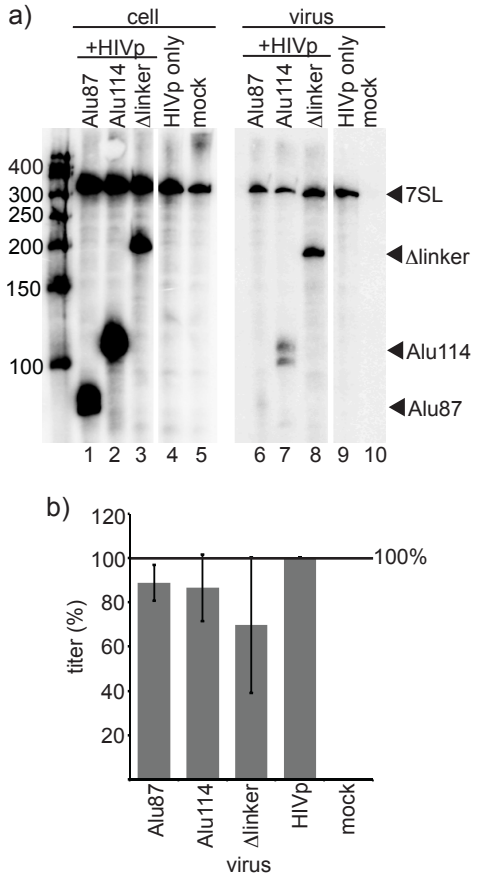

Figure 3-5. Packaging of 7SL derivatives does not have detectable effects on viral infectivity. A) Northern blot of RNA from transfected cells and HIV-1 virus, showing the expression and packaging of 7SL derivatives, visualized with an *Alu* domain probe. B) Titer of 7SL derivative-containing HIVpuro virus, normalized for virion content by an RT assay. Error bars, ±S.D.; n=3 independent experiments.

the genomic RNA content of the HIVp virions by an RPA (Fig. 4d). Viral protein quantification by RT activity confirmed that genomic RNA was proportional to the number of virions present in each sample (data not shown). When normalized to encapsidated gRNA, the total 7SL signal in the S domain derivative-containing virions was 2-4-fold higher than that for the HIVp-only virus (compare lanes 1-3 to lane 4). The amount of total 7SL packaged in the S domain derivative-containing virions was significantly different than the amount of total 7SL packaged in the HIVp-only virions ($p<0.05$). Quantified in Fig. 4e, these results confirm that the S domain derivatives were packaged in addition to endogenous 7SL. Based on 12-14 copies of endogenous 7SL per viral particle, these data indicate that there were ~40 copies of Δlinker per viral particle, ~30 copies of S114 per viral particle, or ~10 copies of S154 per viral particle in addition to the endogenous 7SL.

Packaging of exogenous 7SL, or *Alu* or S domain derivatives, does not affect viral infectivity detectably. Because HIVp genomic RNAs are competent for reverse transcription and integration and contain a puromycin-resistant selectable marker, HIVp puromycin-resistant colony-forming titer was used to determine whether incorporating 7SL derivatives was detrimental to viral infectivity in a single-cycle replication assay. HIVp virions that packaged *Alu* domain or S domain derivatives were used to infect target cells, and the puromycin-resistant titers from a single round of replication were determined. In these experiments, virions were pseudotyped with ecotropic Env and titered on cells that constitutively expressed the ecotropic receptor. Producer cells were co-transfected with HIVp and the *Alu*

domain derivatives Alu87 or Alu114 or the S domain derivative Δlinker. Northern blotting of the RNA content of producer cells and virus was performed to verify exogenous 7SL derivative expression and packaging levels (Fig. 3-5a). Under the transfection conditions used here, these virions contained either 50% of the endogenous 7SL replaced with Alu114, or (in the Δlinker S domain-containing virions) additional 7SL derivatives in 1- to 1.5-fold molar excess of the encapsidated endogenous 7SL.

The puromycin-resistant CFU titers of these viruses are presented in Fig. 3-6b. When normalized to input virus as quantified by RT activity, no significant difference between the titer of virus containing Alu114 or Δlinker (Fig. 3-5b, columns 2 and 3) and those of HIVpuro-only virus (column 4) was detected ($p>0.05$). Similarly, the expression of the non-packaged 7SL derivative Alu87 by producer cells had no effect on the titer of HIVpuro (column 1). These data indicate that the packaging of exogenous 7SL derivatives did not detectably affect viral infectivity under the conditions tested here.

Discussion

We examined the determinants of 7SL packaging by HIV-1 by establishing packaging properties of overexpressed 7SL truncations. 7SL RNA consists of three domains: the left-hand *Alu* domain, the right-hand S domain, and the linker region that separates the two (Fig. 3-1). The results here demonstrated that both the *Alu* and S domains were capable of mediating packaging independently. They also showed that the *Alu* domain competed with endogenous 7SL for packaging, while

the amount of endogenous 7SL remained unchanged when excess S domain was packaged. Some 7SL derivatives that were highly expressed were not packaged at detectable levels, providing information on the determinants of 7SL packaging and confirming that the packaging of 7SL does not result from a random encapsidation of cellular RNAs. These data indicate that there are two mechanisms by which 7SL can become encapsidated: a "competitive" pathway and an "additive" pathway.

A truncation of 7SL corresponding to the entire *Alu* domain, called Alu114, was found to replace some of the endogenous 7SL in HIVp particles. This competition for packaging between Alu114 and endogenous 7SL implies that endogenous 7SL is packaged via the same packaging pathway as Alu114. This competitive pathway is therefore likely to be the endogenous pathway by which HIV-1 recruits 7SL. This suggests that the Gag-proportionate packaging of endogenous 7SL that is characteristic of several retroviruses (236, 237, 302) involves an interaction of 7SL's *Alu* domain with Gag. Furthermore, the decreased packaging of the shorter *Alu* domain truncations, Alu87 and Alu92, and of the Alu114 derivatives in which helix 5c was mutated, suggests that H5 of the *Alu* domain is crucial in the endogenous pathway. The competitive packaging of *Alu* RNAs that lacked the S domain suggests that the endogenous pathway does not require recognition of the S domain by Gag.

The endogenous pathway appears to limit the number of 7SL molecules acquired. Multiples studies have shown that a fixed number (12-16) of 7SL molecules are acquired by HIV-1, and that the packaging of endogenous 7SL occurs in proportion to Gag and independent of the packaging of gRNA (237, 263, 302). The

evidence here that *Alu* domain derivatives replaced endogenous 7SL in the virion implies that exogenously expressed *Alu* domains serve as substrates in the endogenous pathway, which involves a counting mechanism by which an assembling viral particle acquires a certain number of 7SL molecules. In contrast to this, the additive pathway does not appear to involve a counting mechanism because the total number of RNAs in the virus appeared to increase when additive 7SL derivatives were packaged.

The change in phenotype from competitive to additive packaging when the binding site of SRP9/14 was ablated in Alu114 AC suggests that SRP9/14 binding is involved in the endogenous 7SL packaging pathway. Binding of SRP9/14 to 7SL occurs early in SRP biogenesis and is responsible for maintaining 7SL in the nucleolus for subsequent steps in SRP complex formation (52, 144). Thus, the evidence here that 7SL derivatives that cannot bind SRP9/14 were packaged via the additive pathway suggests that location and timing are crucial to the mechanisms of 7SL packaging.

The data that SRP9/14 binding capability is crucial for packaging via the endogenous pathway suggests a possible model for 7SL packaging. In this model, the endogenous, competitive pathway may involve acquisition of a form of 7SL that ordinarily exists transiently, early in its biogenesis, whereas the additive pathway may involve acquisition of 7SL later during SRP biogenesis and in a different location in the cell. This model suggests that the regulated trafficking of endogenous 7SL through the cell may prevent the packaging of endogenous 7SL through the additive pathway. Observations with the SRP9/14 binding site mutant suggested

that the binding of SRP9/14 may transiently produce a three-dimensional structure in helix 5c of the *Alu* domain that is recognized by Gag but that is altered upon binding of the remaining SRP proteins. In this model, Alu114 was packaged exclusively by the endogenous pathway because it could not bind further SRP proteins and therefore was locked in a packaging-competent form, while Alu AC and the S domain truncations were never in the correct conformation.

This model raises the possibility that HIV-1 acquires 7SL in the nucleus. Although fluorescent microscopy visualization of Gag during HIV assembly and evidence that HIV-1 replication does not require Crm1-dependent nuclear trafficking appears inconsistent with this notion, existing work does not rule out Crm1-independent nuclear trafficking, or the possibility that a small subset of HIV-1 Gags may traffic through the nucleus. It is also possible that HIV-1 Gag acquires 7SL in the cytoplasm but before SRP54 has bound 7SL, that expression of HIV-1 changes the subcellular localization of 7SL, or that a fraction of nuclear SRP assembly intermediates intersect with retroviral assembly in some other way.

The effect of 7SL derivative packaging on viral titer was examined to determine whether alterations to the 7SL content of virions affected infectivity. Under the conditions tested here – that is, between approximately 6 and 24 replaced or additional 7SL-type molecules per virion – the packaging of 7SL derivatives had no discernable effect on titer. This was true whether the packaged 7SL derivative was acquired via the endogenous pathway (Alu114) or the additive pathway (Δlinker). These findings suggest that the normal complement of 7SL is not required for HIV-1 infectivity. If 7SL does contribute to viral replication, the lack of a

detectable effect on infectivity when Alu114 replaced some of the endogenous 7SL implies either that the *Alu* domain alone is capable of fulfilling any 7SL-specific roles, or that the remaining endogenous 7SL is capable of fulfilling that role. Similarly, the lack of a detectable effect on infectivity when Δlinker was packaged implies that the packaging of extra 7SL did not destabilize the virion or interfere with viral replication cycle. This lack of evidence for a role of 7SL in the viral life cycle may instead indicate that 7SL packaging results because 7SL is a parasite of retroviruses, much like how *Alu* elements parasitize the L1 retrotransposition machinery (75). However, it is noteworthy that all experiments here were performed under over-expression conditions in 293T cells: conditions known to mask some forms of restriction and other forms of virus-host interactions. Additionally, we did not produce virions that completely lacked 7SL or 7SL derivatives and cannot rule out a role for 7SL in viral replication.

In summary, these studies establish two pathways by which 7SL can be acquired by HIV-1: the endogenous pathway, which involves the *Alu* domain and SRP9/14 binding, and an additive pathway, which occurs in the absence of SRP9/14 binding and which may be dependent on alternative trafficking of 7SL. The additive pathway can result in the packaging of additional 7SL RNA, and the studies here provide evidence that the nature of these additionally packaged RNAs can be artificially manipulated. Our demonstrated ability to manipulate the RNA content of retroviral virions has potentially far-reaching applications as a delivery mechanism for modified RNAs.

Chapter Four

Conclusions, Implications, and Future Directions

In this chapter, I will discuss the data described in Chapters 2 and 3. I will subsequently discuss the implications of these data, and describe experiments that could be performed to further explore the information obtained from the data. I will also discuss some preliminary data I have obtained on subjects related to the main body of my work, and future experiments that could be performed to answer questions that these preliminary data raise.

Discussion

In this thesis, the factors involved in the packaging of 7SL by HIV-1 were investigated. Chapter 2 used minimal VLPs to probe the *trans*-acting packaging factors, and Chapter 3 examined the packaging of exogenously expressed 7SL derivatives to explore the *cis*-acting packaging factors.

The initial experiments examined the packaging of 7SL in minimal VLPs. It was determined that 7SL was retained as a 111 nt fragment corresponding to part of the S domain in minimal VLPs. We called this fragment 7SL remnant, or 7SLrem. 7SLrem was found in minimal VLPs whose only difference from authentic Gag-only particles was that they lacked the NC domain, and in VLPs whose Gag molecules

retained only the CTD of CA and sp1. These data indicate the following: 1) the NC domain is involved in protecting the majority of 7SL from processing; and 2) acquisition of 7SL by minimal VLPs involves CA/sp1.

Furthermore, the precision by which the ends of 7SLrem are cleaved, coupled with the location of the ends in an unpaired bulge in the secondary structure of 7SL, implies that 7SLrem is processed from intact 7SL by an endonuclease. Genomic RNA is often found nicked when released from virions, and the endonuclease that is responsible for the nicking of genome may also be responsible for the processing of 7SL to 7SLrem in ΔNC VLPs. The identity of this endonuclease remains unknown.

To determine the *cis*-acting packaging sequences of 7SL, the packaging of exogenously expressed 7SL derivatives was examined. First, truncations of 7SL were created that corresponded to the complete *Alu* domain (mutant Alu114) and to successively shorter truncations of the *Alu* domain (Alu92 and Alu87). It was determined that the HIV-1 derivative HIVpuro (HIVp) packaged Alu114 robustly, but packaged the successively shorter *Alu* domain truncations successively less well. Furthermore, mutation of helix 5c prevented the packaging of Alu114. Truncations of 7SL were subsequently created that corresponded to the complete S domain (S154) or to 7SLrem (S114 and Δlinker). S154 and S114 were expressed under the U6 promoter, while Δlinker was expressed under the wild-type 7SL promoter. All three S domain derivatives were packaged robustly by HIVp. Therefore, we concluded that both the *Alu* and S domains are capable of mediating packaging.

Interestingly, RNase protection assays revealed that the mechanism of packaging differed between the *Alu* and S domain derivatives. Namely, the *Alu*

domain derivatives competed for packaging with endogenous 7SL, while the S domain derivatives were packaged in addition to 7SL. This suggests that there are two pathways by which HIV-1 can acquire 7SL: the "competitive" pathway and the "additive" pathway. The competitive pathway appears to be the pathway by which endogenous 7SL is acquired, and is therefore also the "endogenous" pathway. The evidence that it is the *Alu* domain derivatives that are packaged via the endogenous pathway indicates that the *Alu* domain is involved in the packaging of endogenous 7SL. The observation that the packaging phenotype of Alu114 changes when its SRP9/14 binding site is ablated indicates that an intact SRP9/14 binding site may be required for packaging via the endogenous pathway. Because SRP9/14 binds 7SL early in SRP biogenesis and is responsible for maintaining 7SL in the nucleolus, the additive pathway may be dependent upon the misregulated trafficking of 7SL derivatives.

Modeling the interactions of 7SL with Gag

Implications

The minimal VLP data suggest that Gag's NC domain interacts with 7SL RNA's *Alu* domain. This appears consistent with recent reports that both Gag and NC co-immunoprecipitate with SRP9 and SRP14 in HEK293 cells (147). (It should be noted that all of the experiments in this dissertation were performed in 293T cells, an HEK293 derivative.) These data, together with the evidence from the packaging of exogenously expressed 7SL derivatives, suggest that the endogenous packaging pathway involves an interaction (direct or indirect) between the NC domain of Gag

and the *Alu* domain of 7SL. Further evidence to support this model comes from Didierlaurent *et al.*, who found that mutations in the ZF motifs of NC resulted in an increase in packaged 7SL (77). This implies that an intact NC domain may be required for the "counting mechanism" that is characteristic of the endogenous pathway.

This model would require that the acquisition of the 7SL that is processed into 7SLrem by minimal VLPs – acquisition that is meditated by CA/sp1 – must be by the additive pathway. However, the data collected so far indicate that the number of 7SLrem molecules per minimal VLP corresponds roughly to the number of 7SL molecules per authentic virion. This, then, would indicate that the "counting mechanism" that is characteristic of the endogenous pathway is functioning in the acquisition of 7SL by VLPs, and that the endogenous pathway can therefore not involve NC (and must involve CA/s1). To resolve this discrepancy, further experiments must be performed.

Future Directions

Initial experiments to determine the packaging mechanism that generates 7SLrem would involve the co-transfection of 7SL derivatives with minimal VLPs. It has been established that certain 7SL derivatives such as S154, as well as the full-length 7SL mutant 7SL10 (data not shown), are packaged by the additive pathway. Thus, if these constructs were to produce 7SLrem in minimal VLPs, this would indicate that 7SLrem is (or at least is capable of being) generated from RNAs packaged by the additive pathway. Conversely, if 7SLrem is generated from RNAs packaged by the competitive pathway, the co-transfection of Alu114 with minimal

VLPs would result in a decrease in 7SLrem per VLP, as Alu114 would replace some of the endogenous, full-length 7SL in the VLP and is itself not capable of generating 7SLrem. Accurate quantification of the amount of virus present would be necessary to draw conclusions, however, and given that minimal VLPs cannot be quantified using an RT assay or an RPA against genomic RNA, the experiments should be carried out using the VLP constructs ΔNCp1 or Zwt, both of which are recognized by an α-p24 (capsid) Western antibody source. Examination of the packaging phenotype of 7SLrem by ΔNC VLPs in the presence of 7SL derivatives would establish the packaging pathway of 7SLrem and thereby establish which regions of Gag are responsible for the two packaging pathways.

Putative roles for 7SL in retroviral assembly

Implications

At the very least, from the data presented here it can be concluded that more than one non-overlapping portion of 7SL (*Alu* and S) are interacting with Gag, and different domains of Gag (NC and CA/sp1) are interacting with 7SL. This allows for a possible model for 7SL's role in retroviral assembly. One unanswered question in retroviral assembly is how ~2,000 Gags, all with identical primary sequences, form different functional subsets. For example, although all NC domains of Gag are thought to bind genomic RNA, in MLV only around 12 form high affinity interactions with the packaging signal of genomic RNA, and the rest form low affinity interactions (212). Similarly, the majority of CA proteins of HIV-1 form hexamers upon maturation, but about 12 pentamers of CA are formed at the ends of the

conical mature core (186). Considering the ability of 7SL to interact with different domains of Gag and the striking similarity between the number of 7SL molecules in HIV-1 (~12/virion), the number of high affinity NC interactions with genomic RNA (~12/virion), and the number of CA pentamers (~12/virion), 7SL may serve as a nucleating agent or similar trigger and cause conformational changes in subsets of Gag.

Another possible role for 7SL during assembly is as a chaperone for Gag. As described in Chapter 1, both the MA and NC domains of Gag are capable of binding RNA, although the MA domain has a greater affinity for the plasma membrane-located phosphoinositide $PI(4,5)P_2$. It is hypothesized that Gag adopts a "folded over" conformation during the journey from the site of translation to the site of assembly, with both the MA and NC domains in contact with RNA, and that the chaperoning by RNA prevents nonspecific membrane targeting (57, 243). Current models propose that genomic RNA is the nucleic acid that chaperones folded-over Gag to the plasma membrane. However, the majority of Gag molecules appear to arrive at the plasma membrane without genomic RNA (155). It is possible that a cellular RNA such as 7SL acts as the chaperone for the majority of Gag. A brief comparison of the sizes of 7SL and Gag in its extended and folded-over conformation reveals that 7SL is large enough to provide a binding site for both ends of Gag in its folded conformation (Fig. 4-1). Because there are only 12-16 molecules of 7SL in a virion compared to the ~2,000 molecules of Gag, this model would necessitate the majority of Gag releasing 7SL during assembly, presumably

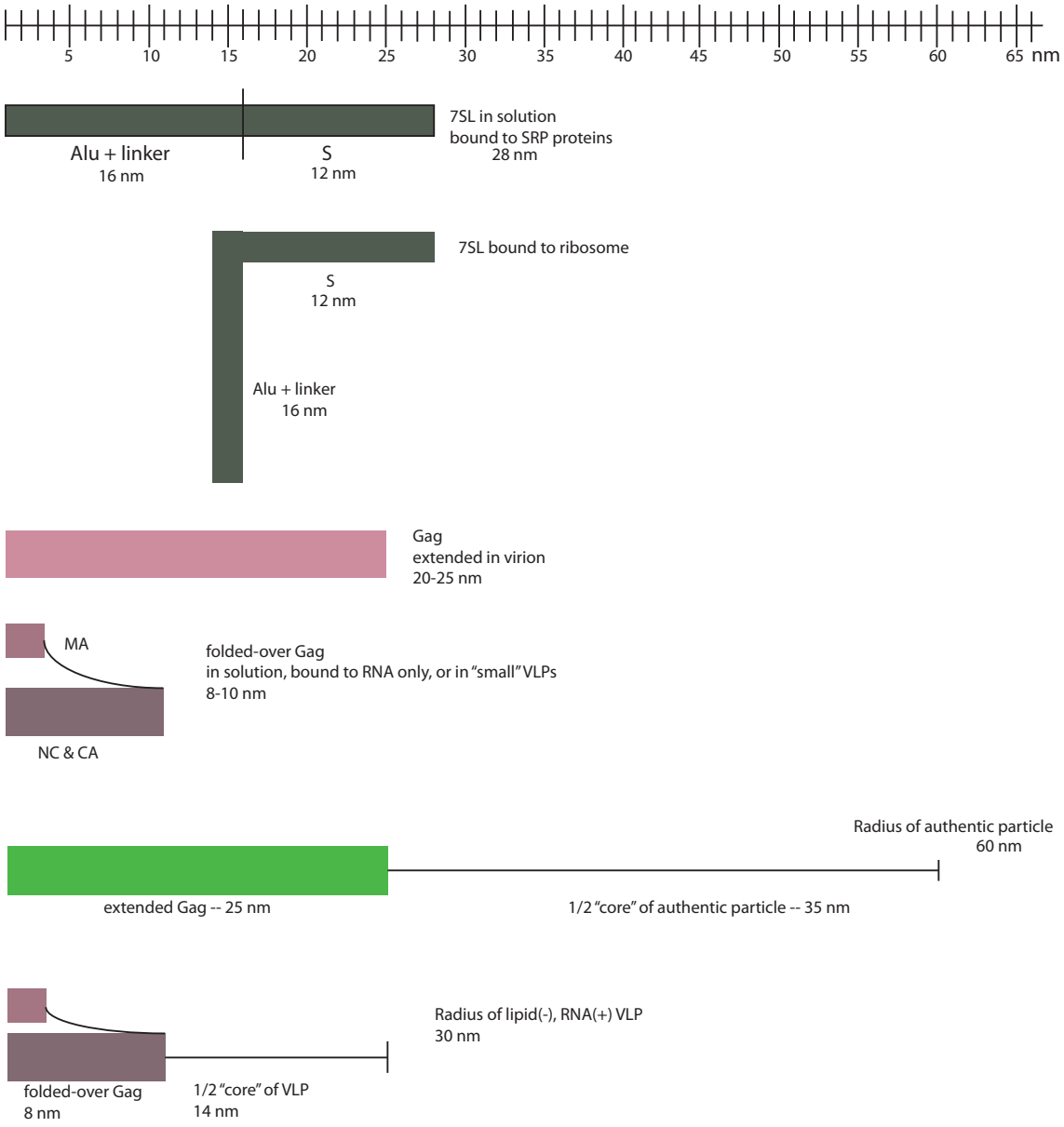

Figure 4-1. Comparison of the sizes of 7SL and Gag. Scale, at top, is in nm. All drawings are to scale. The schematics of the authentic and lipid(-) particles, at bottom, are of the radius, that is, ½ the complete particle.

upon binding to gRNA and PI(4,5)P_2. This model would not preclude the retention of 7SL by a subset of Gags, and so is compatible with the above model in which 7SL nucleates conformational changes in a subset of Gag.

However, the data presented in this thesis do not establish whether 7SL performs any role in retroviral replication, including a role in assembly. Titer data (Fig. 3-5) revealed that the packaging of excess 7SL derivatives (in the form of the Δlinker mutant) did not affect viral infectivity, indicating that the 7SL derivative did not destabilize the viral particle or interfere with entry or subsequent steps in the viral life cycle. Similarly, the replacement of ~50% of the endogenous, full-length 7SL in virions with the *Alu* domain derivative Alu114 did not affect titer, indicating that either a) 7SL has no role in the viral life cycle; b) the role of 7SL in the viral life cycle can be carried out by Alu114; or c) the role of 7SL in the viral life cycle can be carried out by the remaining full-length 7SL. It should be noted that the assays employed in this dissertation looked at the population of virions as a whole, and it is estimated that only around 1 in 8 or fewer virions that enters a cell results in a successful infection (defined as integration of an intact provirus) (78, 177, 301). It is therefore possible that 7SL is contained only in non-infectious particles, or even that the packaging of 7SL itself confers non-infectivity upon a virus.

Preliminary data

To address the putative role(s) of 7SL in retroviral replication, 7SL could be visualized during HIV replication by fluorescence microscopy. Although this would not provide definitive evidence for the putative role(s) of 7SL in retroviral

replication, fluorescence microscopy would provide clues as to when and where 7SL is acquired during HIV-1 assembly, which would help rule in or out various theories as to 7SL's potential role in HIV-1 replication. Fluorescence-based imaging has been widely used to visualize proteins, as the majority of proteins can be tagged with a fluorescent motif such as GFP without appreciable loss of function. However, labeling specific RNAs with fluorescence has proven to be considerably more challenging. The most commonly used technique for fluorescently labeling RNA involves inserting multimerized MS2 protein binding sequences into the RNA of interest (22). The MS2 binding sequence recognizes the MS2 bacteriophage coat protein, which can be tagged with GFP for fluorescence microscopy. The RNA of interest is therefore labeled by association with the tagged MS2 protein rather than being directly labeled itself.

However, this technique is more difficult to perform successfully than it sounds. While this technique has proved useful for labeling mRNAs, which are long, flexible, and relatively unstructured, it may be more difficult to adapt this technique for small, highly structured RNAs such as 7SL. Although the MS2 binding sequence is small (19 nt), it must be in a readily available conformation to enable binding of the tagged MS2 protein. The highly structured character of 7SL and/or its tight interactions with the SRP proteins may preclude this, as our attempts to produce modified 7SL that can be recognized by GFP-tagged MS2 have not been successful (data not shown; performed by Steve King and Eric Garcia). However, our group has not yet successfully imaged mRNAs that contained the MS2 binding sequence, so it

may become possible to recognize 7SL by GFP-tagged MS2 binding once conditions are optimized.

Fluorescence *in situ* hybridization, or FISH, is an alternative mechanism of RNA visualization (256). FISH involves fixing and permeabilizing cells, then exposing them to an oligonucleotide that is complementary to the RNA of interest and that is either tagged with a fluorescent molecule or contains fluorescent nucleotide analogues. I attempted this technique in 293T cells using digoxigenin-labeled oligonucleotides. Although an anti-polyA oligo successfully produced a high signal in the cytoplasm, likely corresponding with mRNA, oligos specific to various portions of 7SL did not produce fluorescent signals higher than background (data not shown). This result probably occurred because FISH requires that the region of RNA that is recognized by the labeled oligonucleotide be accessible to the probe and not base-paired. As there is no region of 7SL longer than ~5 nt that is unpaired in unmodified 7SL, it is unlikely that FISH against wild-type 7SL will be successful.

Future directions

Were I to pursue 7SL visualization further, I would attempt two alternative techniques for fluorescence-based imaging: (a) FISH against a modified 7SL, and (b) engineering 7SL to bind a fluorophore. FISH may succeed if 7SL is modified to introduce an unpaired region into a section of the RNA that is not bound by SRP proteins, such as the linker region. If the unstructured character of this modification can be maintained, it may be recognized by a labeled oligonucleotide. The sequence chosen could be complementary to an oligonucleotide known to function well under FISH conditions, thus improving the chance of successfully producing a visible signal

(280). Success with approach (b) would involve certain RNA sequences that have been shown to induce fluorescence when bound to selective fluorophores such as malachite green or Patent Blue V (9). Modifying 7SL to contain these sequences may enable the visualization of 7SL without relying on labeled probes. An advantage of fluorophore-binding sequences is that they can be used in living cells, without the fixing and staining required for FISH.

Once a protocol for visualizing 7SL has been established, fluorescent microscopy can be used to examine the role of 7SL in the HIV life cycle. For this, single-particle imaging techniques such as stochastic optical reconstruction microscopy (STORM) could enable tracking single molecules of 7SL and prove particularly useful (129, 303), although such techniques are technically challenging. The timing and location of 7SL acquisition by HIV could be examined by co-expressing fluorescently labeled 7SL and GFP-tagged Gag. The location in the cell where 7SL and Gag first co-localize, and any subsequent movement the two molecules may undergo together, would provide information on the step of assembly at which 7SL is acquired. The expression of labeled 7SL derivatives that are not packaged by HIV, such as Alu87, would serve as a negative control.

The fluorescent tagging of genomic RNA, such as by GFP-tagged MS2 proteins as performed by Jouvenet *et al.* (155), would provide further information on the role of 7SL during HIV assembly. For example, if 7SL, Gag, and gRNA all co-localize in the cytoplasm early in assembly, this result may indicate that 7SL serves to nucleate the specific interactions that a subset of Gag undergoes with gRNA. Alternatively, if 7SL co-localizes with a large number of Gag molecules in the cytoplasm but 7SL is

released upon reaching the plasma membrane, this result may indicate that 7SL acts as a nucleic acid chaperone for Gag during its journey to the plasma membrane.

Similarly, viruses released from cells expressing fluorescently tagged 7SL and Gag can be examined to determine if 7SL derivatives are distributed evenly throughout the viral population. A similar protocol has been successfully performed on HIV-1 virions to determine the percentage of virions that contain genomic RNA by tagging Gag with YFP and gRNA with a GFP-tagged MS2 protein (51). To determine whether viral particles containing 7SL are infectious, viral particles containing tagged 7SL and a selectable marker can be used to infect cells at a ratio of approximately one viral particle per cell. During infection, the cells can be examined by fluorescence microscopy to establish which cells receive viral particles containing 7SL. These cells can then undergo selection to determine if they were productively infected, and productive infections can be correlated with the presence or absence of 7SL in the viral particle.

Promoter bias

Background

Three different DNA-dependent RNA polymerases generate cellular RNAs: RNA Polymerase I (Pol I), RNA Polymerase II (Pol II), and RNA Polymerase III (Pol III). Each polymerase recognizes promoters with distinct characteristics, so that promoters can be classified as Pol I, Pol II, or Pol III promoters. mRNAs (that is, genes coding for proteins) are under the control of Pol II promoters, while the major

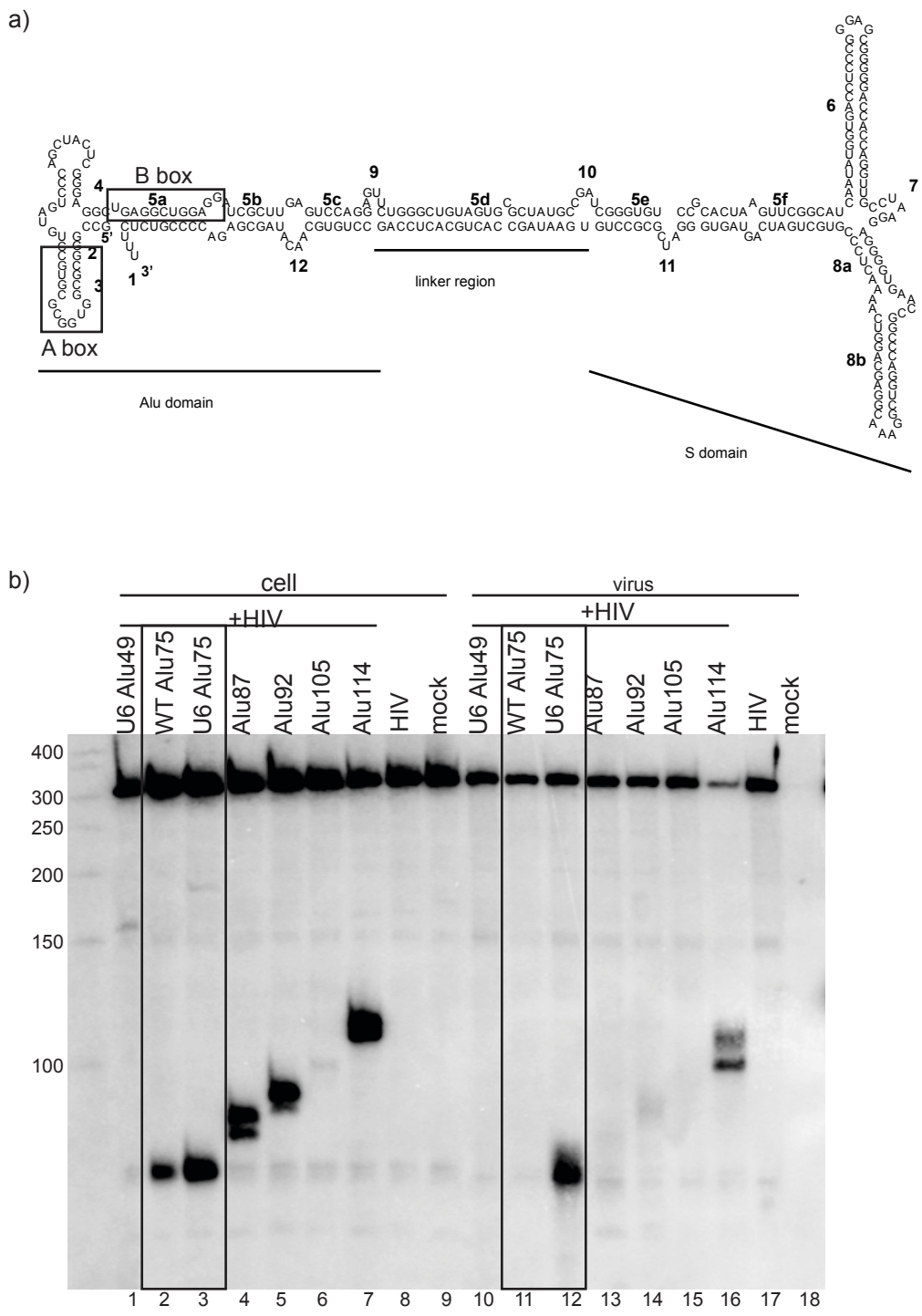

Figure 4-2. (A) The internal promoter elements of 7SL, A box and B box, are boxed. (B) Northern blot of cells co-transfected with *Alu* domain derivatives and HIVpuro and virions produced from these cells. A probe that recognized the *Alu* domain was used.

ribosomal RNA genes are expressed by Pol I promoters, and functional, non-coding RNAs, such as 7SL and U6, are expressed by Pol III promoters.

Pol III promoters are further classified as four types: type I, II, III, and IV (244). All Pol III promoters except type III contain both extragenic and intragenic promoter regions, prohibiting the kind of "cut-and-paste" molecular biology techniques that are often used for protein-coding Pol II genes. As described in Chapter 3, the 7SL promoter is a type IV Pol III promoter and contains two intragenic promoter elements located in the *Alu* domain, the A box and the B box (Fig. 4-2a) (87). Therefore, truncations of 7SL that remove the *Alu* domain or mutations to the promoter sequences within the *Alu* domain cannot be expressed under the 7SL promoter. However, type III Pol III promoters such as the U6 promoter are composed only of extragenic sequences upstream of the transcription start site and thus can be used to express any Pol III RNA.

Preliminary data

Derivatives of 7SL from which the *Alu* domain was removed, such as the S domain derivatives S154 and S114 in Chapter 3, as well as derivatives in which the A box and B box 7SL internal promoter elements were mutated or removed, were expressed using the U6 promoter. Surprisingly, the results indicated that the nature of the promoter used influenced the packaging efficiency of the RNA. For example, the *Alu* domain truncation Alu75 was well-packaged by HIVp when it was expressed by the U6 promoter, but was not packaged when expressed by the 7SL promoter (Fig. 4-2b, compare lanes 11 and 12). This suggests that there exists promoter bias in the population of RNA that is selectively packaged by HIV-1.

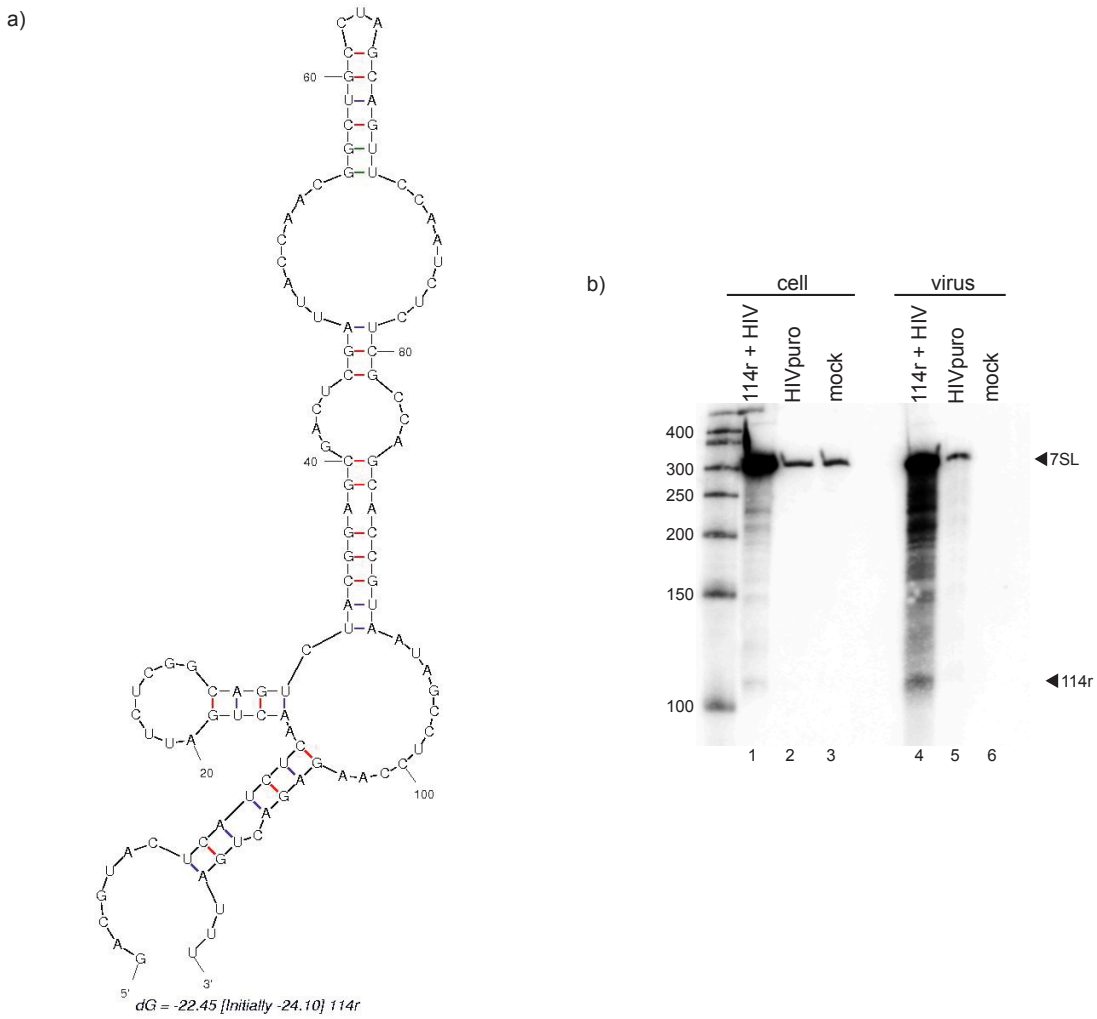

Figure 4-3. (A) 2-dimensional schematic of 114r. (B) Northern blot of cells co-transfected with 114r and HIVpuro and virions produced from these cells. Probes that recognized the *Alu* domain of 7SL and 114r were used.

To determine whether expression by the U6 promoter conferred packaging selectivity on RNA, a random nucleotide sequence was generated, using an online random nucleotide sequence generator (http://www.faculty.ucr.edu/~mmaduro/random.htm), and placed under the U6 promoter. This sequence, called 114random or 114r, was 114 nt in length, had the same GC content as 7SL, and was predicted to fold into a shape that was dissimilar to 7SL or 7SL derivatives by the software Mfold (http://mfold.rna.albany.edu/?q=mfold/) (Fig. 4-3a). 114r was both expressed in cells co-transfected with HIVp and packaged in HIVp virions, indicating that expression under the U6 promoter confers packaging selectivity on Pol III-promoted RNAs (Fig. 4-2b). Because of these observations, we felt that the packaging of the S domain constructs S154 and S114, designed to examine the influence of the S domain on 7SL packaging, might be influenced by their expression by the U6 promoter. Therefore, we designed the 7SL derivative Δlinker, which was expressed by the 7SL promoter, to ensure that the conclusions drawn from the packaging of the S domain constructs were not due to promoter bias. Furthermore, the ability of HIV to package artificially designed RNAs expressed by the U6 promoter has far-reaching applications that will be discussed below.

Future directions

The apparent selectivity in packaging by HIV toward RNA expressed by a certain promoter should be examined to further establish the characteristics necessary for selective packaging of RNAs by HIV. Previous work in MLV identified an apparent bias in packaging toward Pol III over Pol II and Pol I products (236). It

is unclear whether this packaging bias is due to the identity of the RNAs that are selectively packaged or a shared feature of Pol III transcription – for example, the 5' triphosphate of Pol III transcripts or a shared trafficking pathway. To address this, small Pol II transcripts could be placed under a Pol III promoter, and a packaged Pol III product such as 7SL could be placed under a Pol II promoter, and their packaging phenotype examined. Ideally, these RNAs could also be fluorescently tagged (see above) so their trafficking could be examined.

LysRS and 7SL acquisition

Implications and background

The data presented in Chapter 2 on the presence of 7SLrem in minimal VLPs suggest that CA/sp1 is involved in the acquisition of 7SL. This result was surprising because the CA-CTD and sp1 do not contain known RNA binding motifs, nor has CA/sp1 been shown to interact with RNA *in vitro* or *in vivo*. This suggests that the interaction between CA/sp1 and 7SL may be indirect: that is, that 7SL may interact with CA/sp1 via a protein intermediate. One possible candidate for such a role is the SRP heterodimer SRP9/14. However, recent evidence suggests that SRP9/14 interacts with NC, although this evidence does not rule out interactions between SRP9/14 and CA (147). Furthermore, SRP9/14 binds the *Alu* domain of 7SL, and it is the *Alu* domain that is degraded in ΔNC VLPs. This may be evidence that the *Alu* domain of 7SL interacts with the NC domain of Gag, which would suggest that SRP9/14, as the *Alu* domain-binding heterodimer, also interacts with NC.

Another candidate for a possible protein intermediary between 7SL and Gag is the lysyl-tRNA synthetase, LysRS. As described in the introduction, LysRS is

packaged by HIV as part of the mechanism by which HIV acquires its primer tRNA, $tRNA^{Lys,3}$ (146, 199). LysRS encapsidation is dependent upon the CTD of CA, making it an attractive candidate for the role of putative protein bridge between 7SL and CA-CTD (167, 168). Furthermore, when the amount of LysRS in HIV-producing cells is decreased by RNAi or increased by overexpression, the amount of LysRS in the virion decreases or increases accordingly (98, 119). This result provides a simple way to test whether or not LysRS influences the packaging of 7SL: namely, manipulate the LysRS content of the virion – either by knocking down or overexpressing LysRS in the virus-producing cell – and determine whether the 7SL content of the virion changes proportionally.

Future directions

To perform these experiments, a plasmid expressing His-tagged LysRS and verified siRNAs against LysRS were obtained (98, 119). However, neither knockdown nor overexpression were successfully achieved under the tested conditions. The overexpression and knockdown of LysRS should be optimized so that the amount of 7SL can be determined in virions that contain more or fewer LysRS molecules. In addition to examining the effect of LysRS packaging on 7SL encapsidation, the interactions between 7SL and viral and/or cellular proteins can be examined by crosslinking. The technique of crosslinking and immunoprecipitation (CLIP) has been effectively used to probe interactions between proteins and RNA (70, 107). To perform protein/RNA CLIP, a sample is subjected to crosslinking by chemical reagents or UV light, and the protein of

interest is immunoprecipitated by an antibody. The identity of the substrate's binding partners can be identified by blotting, RT-PCR, or sequencing.

To use CLIP to examine interactions between 7SL and viral and/or cellular proteins, both virions and cells that are producing HIV virions can be subjected to CLIP. Because 7SL is ultimately recruited by Gag, an anti-Gag antibody can be used for the initial immunoprecipitation. Mass spectrometry can then be used to identify any other proteins in the immunoprecipitated complex. Although careful optimization would be necessary, CLIP is a promising technique for directly studying the interactions of 7SL and viral and cellular proteins during and after assembly, and could be used to determine the identity of the putative protein that mediates the interaction between 7SL and Gag.

Y RNA packaging

Background

One such difference in the small RNA profile of HIV and MLV is the selective packaging of Y RNAs: while MLV packages the murine Y RNAs mY1 and mY3 at levels of enrichment comparable to 7SL (236) and acquires Y RNAs early in their biogenesis (104), wild-type HIV does not package hY1 or hY3 at appreciable amounts (302, 309). Two models that can be proposed to explain these differences are 1) HIV successfully excludes Y RNAs, or 2) MLV selectively acquires Y RNAs. We sought to distinguish between these models by examining the interactions between HIV and Y RNAs.

Preliminary data

To determine the effect of genomic RNA packaging on Y RNA packaging, we examined the packaging of hY1 in HIV VLPs that lacked gRNA. Previously, Khan *et al.* (163) discovered that hY1 and hY3 were packaged by a genome(-) HIV VLP generated by expression of a provirus construct with mutations in Ψ, but that hY1 and hY3 were not packaged by a genome(-) VLP generated by expression of a provirus with mutations in the zinc finger motifs of Gag. From this, the authors concluded that Y RNA packaging is mediated by the same regions of NC as gRNA, but that Y RNAs are excluded from Ψ(+) HIV particles because NC has a higher affinity for gRNA (163). However, when we revisited this question using a similar HIV-1 Ψ(-) construct, ΔR8.2, we found that this genome(-) VLP did not contain detectable amounts of hY1 (Fig. 4-4a, lane 2). ΔNC and ΔMA VLPS also did not package hY1 (Fig. 4-4a, lanes 9 and 10). Strikingly, however, the Gag-only construct hGag did package hY1 robustly (Fig. 4-4a, lane 7).

hGag is a codon-optimized Gag-only construct that produces Gag-only VLPs (that is, they lack GagPol) (138). hGag also does not encode any of HIV's accessory factors. As MLV also does not encode accessory factors, we hypothesized that an accessory factor of HIV might be excluding the Y RNAs from encapsidation. Seemingly consistent with this hypothesis, the Ψ(-) construct employed by Khan *et al.* is ΔNef, whereas our Ψ(-) construct, ΔR8.2, expressed Nef. We therefore hypothesized that Nef might function as an exclusion factor for Y RNAs during HIV assembly. To test this hypothesis, we examined the Y RNA packaging phenotype of

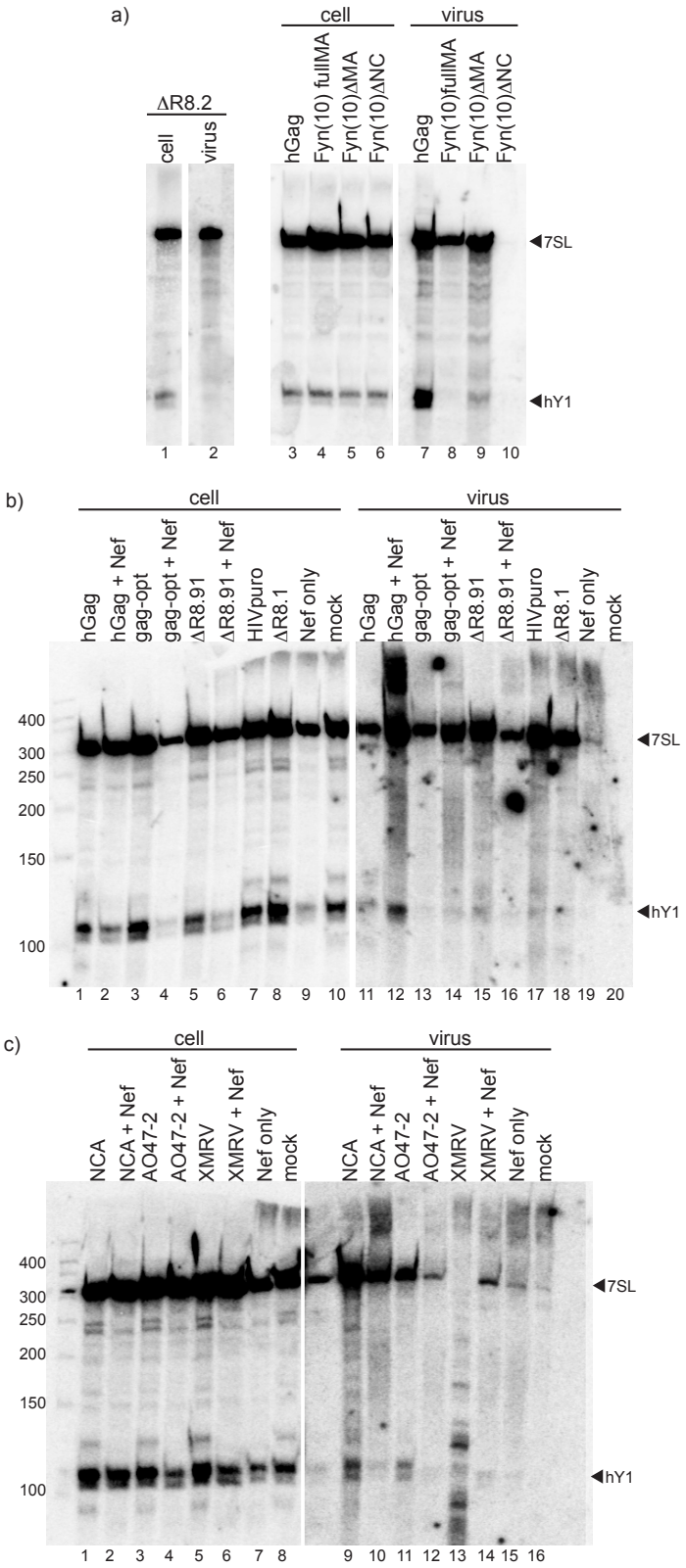

Figure 4-4. Northern blots of transfected cells and VLPs produced from these cells. (B) HIV viruses; (C) MLV viruses. Probes that recognized the *Alu* domain of 7SL and hY1 were used.

several ΔNef HIV constructs obtained from the NIH AIDS Research and Reference Reagent Program (https://www.aidsreagent.org/index.cfm) as well as the Y RNA packaging phenotype of MLV produced by cells co-transfected with a Nef-producing plasmid. Northern blotting revealed that hY1 was not packaged to detectable levels by ΔR8.91, a Ψ(-) ΔNef HIV VLP (342), suggesting that Nef does not prevent the packaging of hY1 (Fig. 4-4b). Similarly, hY1 was packaged by hGag produced in the presence of exogenous Nef (102) (Fig. 4-4b) and by MLV produced in the presence of exogenous Nef (Fig. 4-4c). We therefore concluded that Nef does not influence the packaging of Y RNAs by retroviruses.

Surprisingly, we discovered that, in contrast to hGag, gag-opt, another Gag-only particle (102), did not package hY1 (Fig. 4-4b). Because both hGag and gag-opt are Gag-only constructs, we concluded that accessory factors have no influence on Y RNA packaging. Instead, the differences in Y RNA packaging must be due to a difference between the RNA and/or the protein of hGag and gag-opt. hGag and gag-opt are both Rev-independent, codon-optimized Gag-only constructs. However, their amino acid sequences are only about 80% similar, and their RNA sequences are about 60% similar. Furthermore, while both are expressed under the CMV promoter, the hGag plasmid contains an intron between the promoter and the hGag coding region, and the 5' and 3' UTRs of both constructs have no homology. Interestingly, hGag is HIV subtype B, the same subtype as the typical laboratory strains of HIV, while gag-opt is subtype C (102, 138). Yet, it is gag-opt that exhibits the same packaging phenotype as the other laboratory strains, and hGag that does not. This suggests that the difference in packaging may be due to differences in

trafficking, rather than the sequence of the protein. Although both hGag and gag-opt are Rev-independent, the pathway by which they exit the nucleus has not been established, and it is possible that they are trafficked through different pathways.

Because RNA trafficking pathways can influence viral assembly (149, 150, 293), we first sought to determine if the Y RNA packaging phenotype mapped to the 5' UTR, the 3' UTR, or the protein coding region. We created 5' and 3' UTR swaps, detailed in Fig. 4-5a. Northern blotting of the RNA content of the virions produced by these constructs revealed that the packaging of hY1 mapped to the protein coding region (Fig. 4-5b, see particularly lane 11).

Future Directions

The creation of chimeras of the protein coding regions of hGag and gag-opt should be performed to further narrow down the region of the RNA and/or protein that is responsible for the difference in Y RNA packaging phenotype. There are only ten amino acid differences between hGag and the Gag of ΔR8.2, the Ψ(-) construct that does not package hY1 (in CA, sp1, and NC), approximately a 2% difference. This suggests that the difference in Y RNA packaging between hGag and other type B HIV strains, or between hGag and gag-opt, is not due to the sequence of the protein, although it may be due to a difference in the trafficking of the protein. The trafficking of the RNA may also play a role in the difference in Y RNA packaging between hGag and ΔR8.2, as hGag is Rev-independent, while ΔR8.2 is Rev-dependent. Similarly, the trafficking pathways of the RNAs may play a role in the different phenotypes of hGag and gag-opt. Therefore, live-cell imaging of the proteins (by creating GFP fusion proteins) and RNA (by the addition of MS2 binding

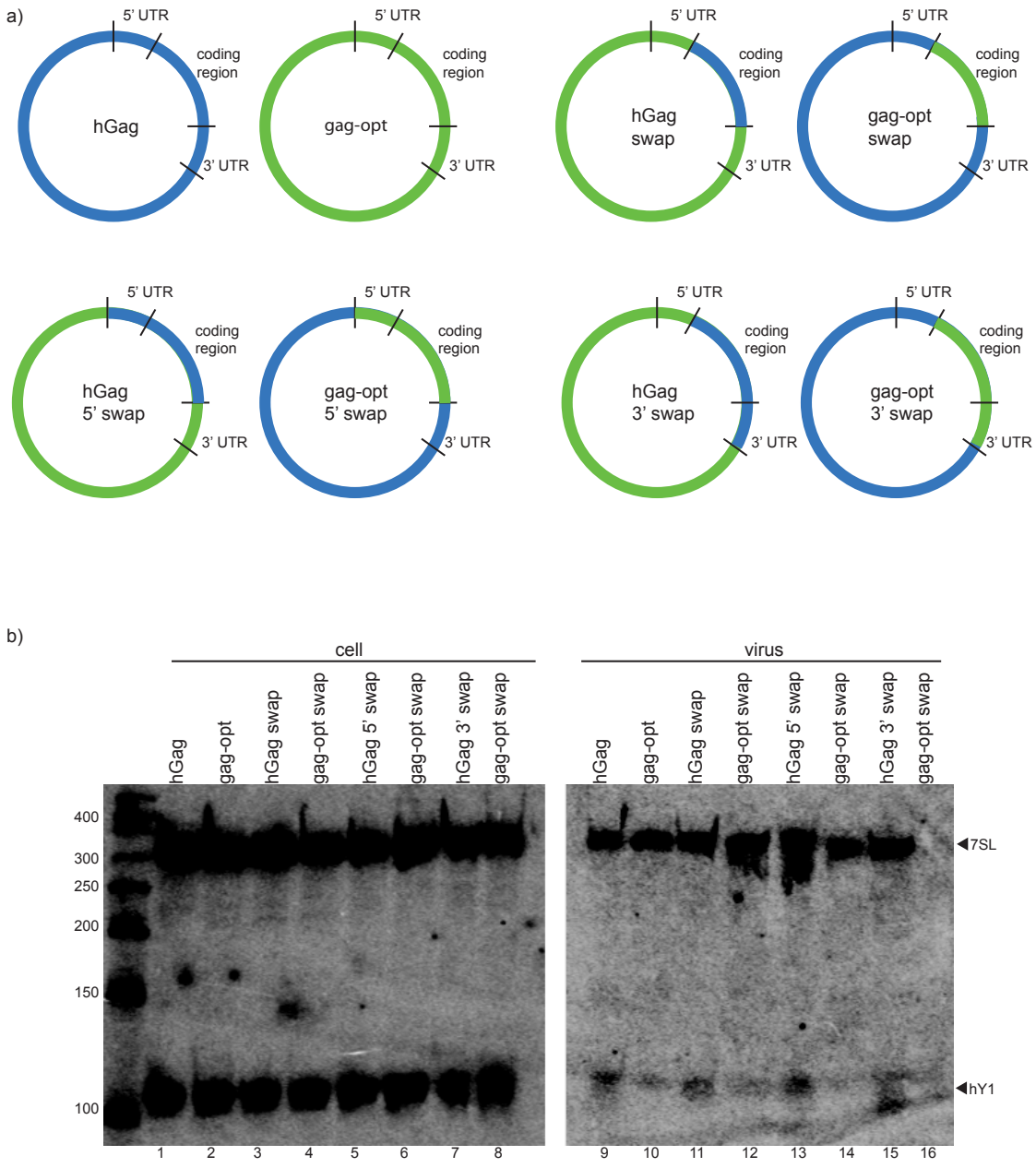

Figure 4-5. (A) Schematic of the hGag and gag-opt UTR swaps. (B) Northern blot of cells transfected with the UTR swaps and VLPs produced from these cells. Probes that recognized the *Alu* domain of 7SL and hY1 were used.

sequences) during assembly should be undertaken to provide evidence of possible differences in trafficking pathways between the two constructs.

IFN stimulation by Pol III RNA

Background

The discovery that the Gag-only VLP hGag packages hY1 leads to the formation of a hypothesis involving packaged cellular RNAs and the innate immune response to HIV. As described in the Introduction, HIV does not usually provoke a strong IFN response. However, the original paper on hGag described the construct as highly immunogenic when DNA plasmids coding for hGag were injected into mice (138). The packaging of Y RNAs by hGag provides a possible explanation for how this could be achieved: Y RNAs contain the 5' triphosphate motif recognized by the cytoplasmic innate immune sensor RIG-I, and the highly ordered three-dimensional structure recognized by the related sensor MDA5. Coupled with the absence of the anti-IFN accessory factors Vif and Vpr (46, 80), recognition of unbound Y RNAs after uncoating via RIG-I and/or MDA5 may explain the increased immune response to hGag.

Future Directions

Our demonstrated ability to manipulate the RNA content of HIV virions provides a useful tool to test this hypothesis. HIV virions can be produced under such conditions that they package excess small, highly structured, Pol III-produced (and thus 5'-triphosphorylated) RNAs. These virions can be used to infect a cell line known to produce IFN in response to the stimulation of RIG-I and MDA5 such as

dendritic cells (159, 160), and the amount of IFN produced can be measured by ELISA assay. These experiments have the potential to further clarify the role of the innate immune system in the immune response to HIV.

The influence of 7SL on APOBEC3G packaging

Background

As described in Chapter 1, opinion is currently divided over whether the packaging of the innate antiviral protein APOBEC3G is mediated by 7SL or genomic RNA. Some studies have found that A3G is not packaged in genome(-) particles (163, 164), while other studies have found that A3G is packaged in genome(-) particles (310). Similarly, some studies have found that decreasing the amount of 7SL in HIV particles results in a proportional decrease in A3G packaging (309, 310), while other studies have failed to replicate these results (10, 163). Interestingly, one study has determined that the average number of A3G molecules in ΔVif HIV-1 particles expressed in primary peripheral blood mononuclear cells is 7 (329). The rough similarity of this number to the average number of 7SL molecules per HIV-1 particle (12-16) is provocative. Increasing the expression of A3G has been shown to increase its packaging (306); whether such an increase in A3G packaging affects the number of 7SL molecules packaged remains to be determined.

Future directions

The packaging of exogenous 7SL by HIV virions can be used to explore the packaging of APOBEC3G. If co-transfections are carried out in an A3G(+) cell type, virions containing exogenously expressed 7SL derivatives (for example, Alu114,

S154, or the full-length 7SL derivative 7SL10) can be examined for A3G encapsidation. If 7SL mediates the packaging of A3G, it would be expected that the amount of A3G packaged by HIV-1 would increase when extra exogenous 7SL derivatives are packaged. These data may help to resolve the discrepancy between studies that sought to determine the RNAs involved in packaging A3G.

Aptamer delivery

Background

The ability to manipulate the RNA content of retroviral virions has potential applications for a virus-based drug delivery mechanism. Virus-like particles (VLPs) are ideal drug delivery vehicles because viruses have evolved to deliver biological molecules to the cytosol of cells (327). VLPs retain this functionality but, because of the absence of genome in the VLP, are no longer replication competent and will not produce an infection. Furthermore, by carefully selecting the envelope protein(s) of the VLP, VLPs used as delivery vehicles can be targeted to specific cell types (273, 333).

One class of drugs ideally suited for viral delivery are aptamers. Aptamers are small nucleic acids, usually RNA or modified RNA, that bind to a specific ligand. Aptamers are designed through a process of *in vitro* forced evolution called "systematic evolution of ligands by exponential enrichment" (SELEX) (85, 287, 304). A primary advantage of aptamers over traditional small molecule drugs is their ease of synthesis: as nucleic acids, they can be coded into DNA and produced by standard *in vitro* transcription reactions. An aptamer used for the treatment of macular

degeneration (brand name Mucagen) has already met with FDA approval, and aptamers designed to treat blood clotting, diabetes and cancer are in clinical trials (36, 230). Aptamers have even shown promise as anti-retrovirals (127). Virus-derived delivery vehicles are perfectly suited for aptamer deliver because viruses have evolved to encapsidate nucleic acid and deliver that nucleic acid to cells.

Preliminary data

To determine if HIV VLPs could package an exogenously expressed aptamer, we examined the packaging of the 54-base aptamer SRB2m. The SRB2m aptamer has been shown to bind the dye Patent Blue V and induce fluorescence, thus making the aptamer easily assayable (9, 132, 320). We designed two constructs that express the SRB2m aptamer (Fig. 4-6a). For the construct "SRB2m", the SRB2m sequence was placed directly under the control of the U6 promoter, and five Ts were added immediately 3' of the SRB2m coding region to terminate transcription. For the construct "S154/SRB2m", the SRB2m sequence replaced helices 6, 7, and 8 of the 7SL derivative S154. S154/SRB2m was also under the control of the U6 promoter.

The SRB2m constructs were co-transfected into 293T cells with plasmids that produced either authentic HIV (HIVpuro) or Gag-only VLPs (Gag/h). The RNA content of the co-transfected cells and the virions produced from these cells was examined by northern blotting (Fig. 4-6b). The SRB2m aptamer itself was not detectable in virions, but the S154/SRB2m RNA was robustly packaged, possibly due to its increased length (Fig. 4-6b). U6-promoted RNAs of around 50 nt, such as Alu49, have also been seen to be packaged at reduced levels by HIVp (data not shown). This indicates that HIV VLPs can package exogenously expressed aptamers.

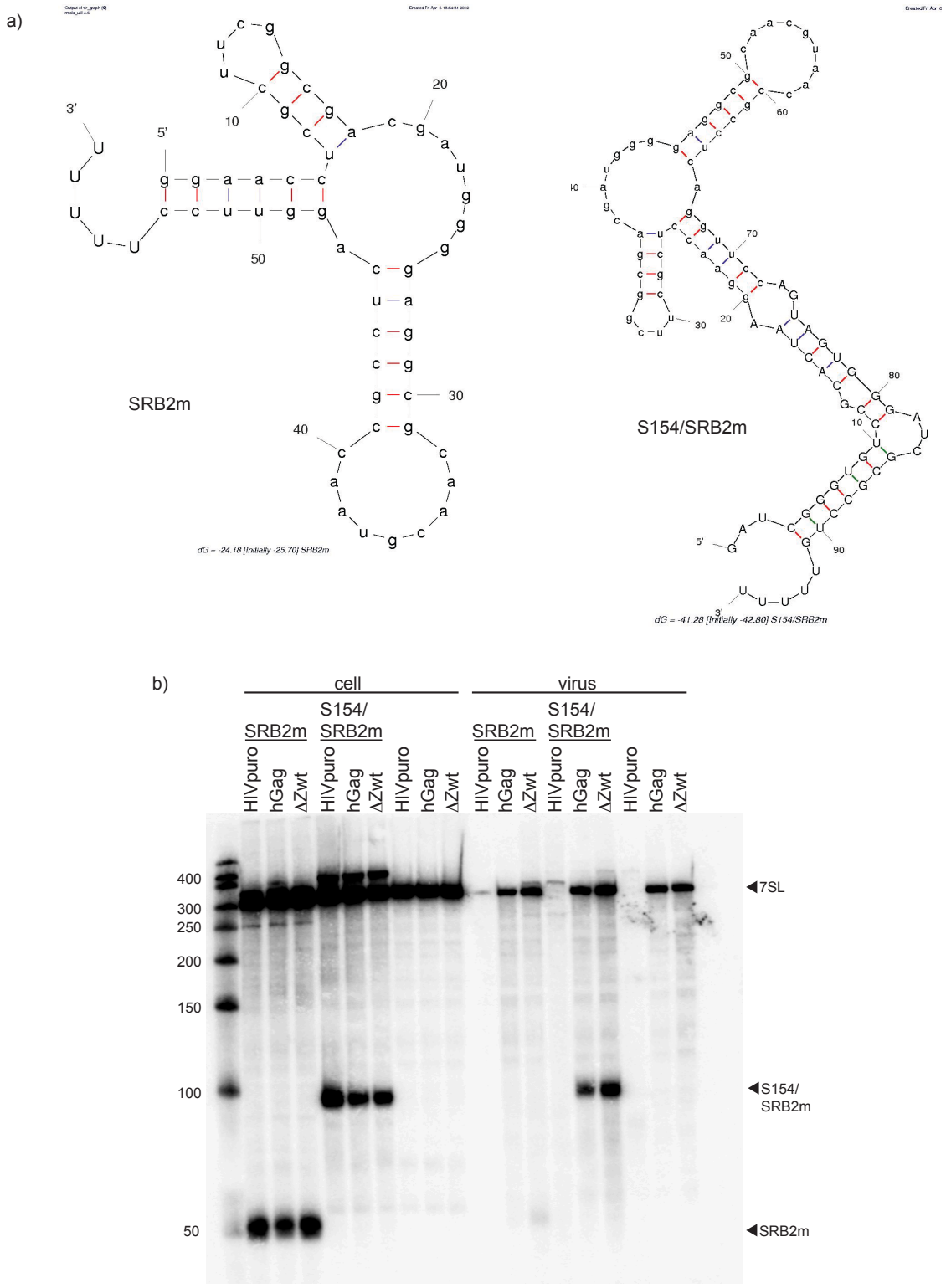

Figure 4-6. (A) 2-D schematics of SRB2m and S154/SRB2m. (B) Northern blot of cells co-transfected with SRB2m constructs and virus constructs, and virions produced from these cells. Probes against the *Alu* domain of 7SL and SRB2m were used.

Future directions

Further experiments are necessary to establish whether virions containing aptamers can deliver the aptamers into target cells, and whether those aptamers retain their functionality after delivery. Because the SRB2m aptamer induces fluorescence when bound to the dye Patent Blue V, the successful delivery of the SRB2m-containing 7SL derivative could be measured by fluorescence microscopy.

Influence of genome on packaging

To further establish conditions under which exogenously expressed cellular RNAs could be packaged, we examined the packaging of 7SL derivatives by genome(-) particles. We discovered that the Ψ(-) construct ΔR8.2 packaged 7SL derivatives that were not packaged by the Ψ(+) construct HIVpuro. For example, the shorter *Alu* domain truncations Alu87 and Alu92 were packaged by ΔR8.2, whereas they were not packaged by HIVpuro (Fig. 4-7a, compare lanes 8 and 9 to lanes 20 and 21). ΔR8.2 also packaged higher amounts of the U6-promoted constructs S154 and S114 than HIVpuro (Fig. 4-7b, compare lanes 5 and 6 to 13 and 14): around 80% of the total 7SL RNA (mutant and endogenous) in ΔR8.2 virions was the S domain derivatives, whereas around 50% of the total RNA in HIVpuro virions was the S domain derivatives. The constructs ΔR8.2 and HIVpuro are similar enough that the primary difference between them is the presence or absence of genome. This suggests that some small cellular RNAs are packaged in competition with genome.

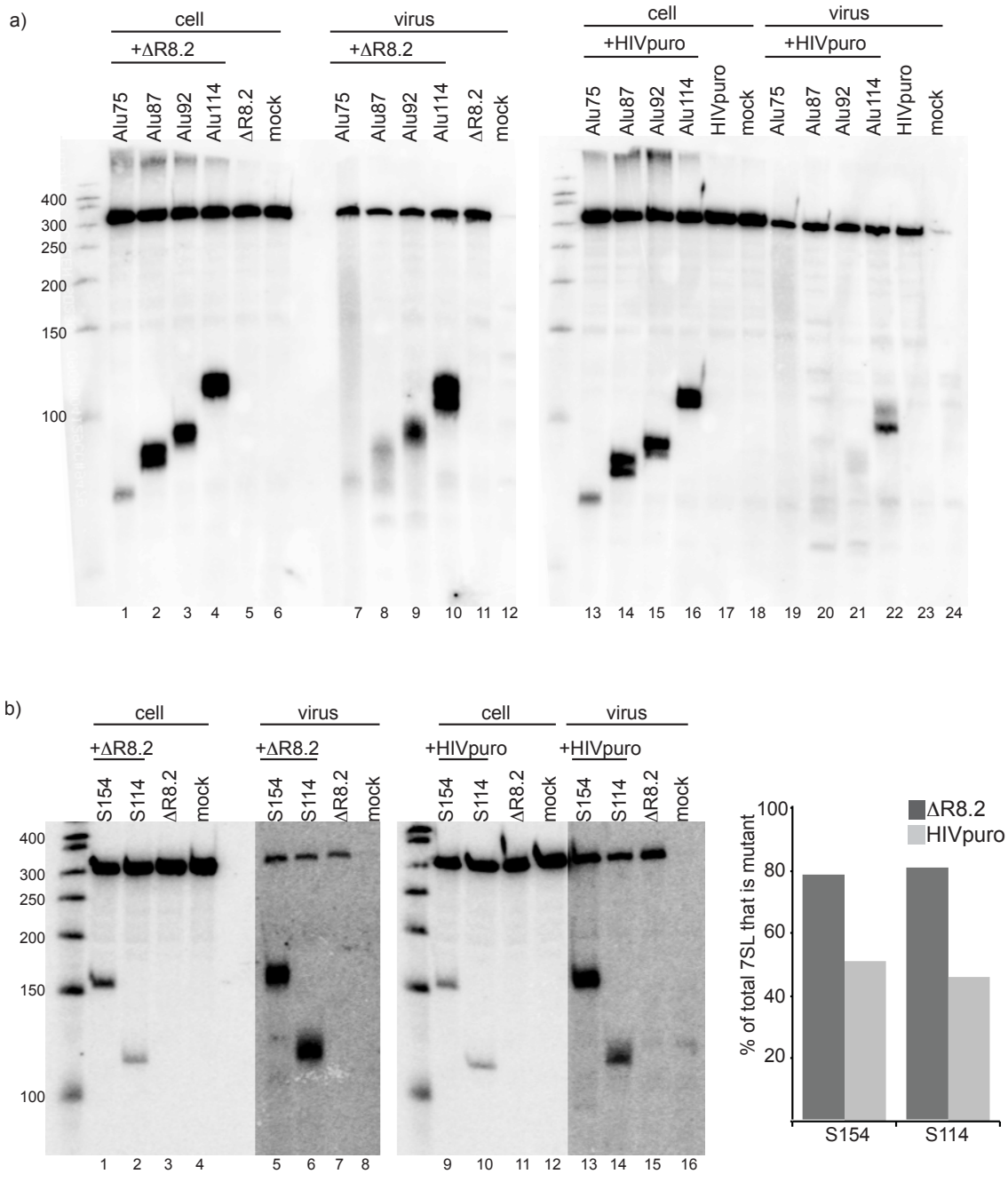

Figure 4-7. Northern blots of cells co-transfected with 7SL derivatives and the Ψ(-) construct ΔR8.2 and the Ψ(+) construct HIVpuro and virions produced from these cells. Probes used were against the *Alu* domain of 7SL (A) or the S domain of 7SL (B).

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