NOTE TO USERS

The Role of Lysyl-tRNA Synthetase in Selective Packaging of $tRNA^{Lys3}$ into HIV-1

By

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Your file Votre référence
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To my parents, Jamshid and Parvin Javanbakht

For their patience, constant support, and encouragement

Abstract

During HIV-1 assembly $tRNA^{Lys}$ isoacceptors are selectively packaged into HIV-1. One of the $tRNA^{Lys}$ isoacceptors, $tRNA^{Lys3}$ serves as a primer for the reverse transcriptase-catalyzed synthesis of minus strand, strong stop cDNA. Lysyl-tRNA synthetase (LysRS) a $tRNA^{Lys}$-binding protein is also selectively package into HIV-1, making it a candidate for being the signal which targets $tRNA^{Lys}$ for incorporation into viruses. We have tested whether the cytoplasmic $tRNA^{Lys}$/LysRS interaction is required for selective packaging of $tRNA^{Lys}$ into HIV-1 by constructing anticodon mutations within the anticodon domain of $tRNA^{Lys3}$. The anticodon is the major binding domain for $tRNA^{Lys}$/LysRS interaction. We found that the ability of $tRNA^{Lys3}$ to bind to LysRS, and be aminoacylated was directly correlated with its ability to be incorporated into HIV-1.

However it was not clear from these results if in addition to the tRNALys/LysRS interaction, tRNA aminoacylation was required for selective packaging of $tRNA^{Lys3}$. To investigate this, we constructed and expressed two mutant LysRS species. One has lost its aminoacylation activity because of a deletion within the catalytic core. The other mutant LysRS has a reduced ability to bind to $tRNA^{Lys}$, because it contains a deletion within $tRNA^{Lys}$ binding site. This mutant LysRS does not have the ability to facilitate $tRNA^{Lys}$ packaging into the virus, while the mutant LysRS which binds to $tRNA^{Lys}$, but fails to aminoacylate it, does facilitate $tRNA^{Lys}$ packaging, indicating that $tRNA^{Lys}$ aminoacylation is not required for its packaging into viruses.

LysRS is carried into the viruses by its interaction with Gag. We have mapped the sites of interaction between HIV-1 Gag and LysRS using *in vivo* and *in vitro* techniques. We find that Gag sequences within the C-terminal domain of CA which contains the CA dimerization site, interact with LysRS sequences which include motif 1, which contains the LysRS dimerization site.

Résumé

Durant l'assemblage du VIH, l'ARN de transfert Lysine (ARNt ^{Lys}) est incorporé de manière sélective dans le virion. Un des isoaccepteurs de l'ARNt ^{Lys}, l'ARNt ^{Lys3} sert d'amorce pour la synthèse du brin négatif par la transcriptase inverse. L' ARNt ^{Lys} synthètase, également intégré dans la particule virale, représente un candidat pour expliquer l'incorporation de l' ARNt ^{Lys} . Afin d'évaluer la nécessité de l'interaction dans le cytoplasme entre l'ARNt ^{Lys} et l'ARNt ^{Lys} synthetase dans le contexte d'incorporation sélective, nous avons effectué des mutations dans l'anticodon de l'ARNt ^{Lys3}; l'anticodon est le principal domaine d'interaction entre l'ARNt ^{Lys} et l'ARNt ^{Lys} synthetase. Nous avons découvert que l'habileté de la liaison entre ces deux molécules et l'aminoacylation de l'ARNt ^{Lys} corrélait directement avec la capacité de son intégration dans le VIH.

Cependant, il demeurait incertain si, en plus de l'intéraction entre l'ARNt ^{Lys} et l'ARNt ^{Lys} synthetase, l'aminoacylation de l'ARNt était nécessaire pour son incorporation sélective.

Afin d'y répondre, deux mutants ARNt ^{Lys} synthetase furent construits et exprimés. L'un deux possède une délétion dans le domaine catalytique et ne peut aminoacetyler, l'autre, en raison d'une mutation dans le site de liaison avec l'ARNt présente une affinité moindre pour l'ARNt ^{Lys3}. Ce mutant ARNt ^{Lys} synthetase a perdu sa capacité de faciliter l'emballage de l'ARNt ^{Lys3} dans le virus tandis que le mutant ARNt ^{Lys} synthetase qui se lie à l'ARNt ^{Lys} mais échoue

à l'aminoacetyler demeure efficace pour favoriser l'intégration de l' ARNt ^{Lys} dans les virions en formation. L'aminoacetylation de l'ARNt ^{Lys} n'est par conséquent pas nécessaire dans cette incorporation spécifique.

Le transport de l'ARNt ^{Lys} synthetase dans le virion s'explique par son interaction avec Gag. Nous avons repertorié les sites de ces interaction entre Gag et l'ARNt ^{Lys} synthetase en utilisant des techniques à la fois *in vivo* et *in-vitro*. La séquence de Gag au sein de l'extrémité C terminal de la caspide qui contient le site de dimerisation de gag, interagit avec la sequence de l'ARNt ^{Lys} synthetase qui inclut le motif 1 et possède le site de dimérisation de l'ARNt ^{Lys} synthetase.

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Preface:

In accordance with the guidelines for thesis preparation, the candidate has exercised the option of writing the thesis as a manuscript-based thesis. A General Introduction is presented in Chapter 1. Chapters 2-4 are original manuscripts that has been submitted for publication. Each chapter contains its own abstract, introduction, experimental procedure, results and discussion. A general discussion to the thesis has been included in chapter 5. In chapter 6, the candidate lists the original contributions to the scientific community. In order to bridge the connecting papers, Chapters 2 to 4 each contains a preface. The manuscripts presented in the thesis are the following:

Javanbakht, H., S. Cen, J. Sadaatmand, K. Musier-Forsyth, and L. Kleiman. 2003, *submitted*. The ability of Wild type and mutant lysyl-tRNA synthetase to facilitate $tRNA^{Lys3}$ incorporation into virus.Journal of Virology.

Javanbakht, H., R. Halwani, S. Cen, J. Sadaatmand, K. Musier-Forsyth, H. G. Gottlinger, and L. Kleiman. 2003, *in press*. The interaction between HIV-1 Gag and human lysyl-tRNA synthetase during viral assembly. J. Biol. Chem.

Javanbakht, H., S. Cen, K. Musier-Forsyth, and L. Kleiman. 2002. Correlation between $tRNA^{Lys3}$ aminoacylation and incorporation into HIV-1. J.Biol. Chem. 277:17389-17396.

The candidate was responsible for all the work presented in this thesis but would like to acknowledge the contribution of colleagues in Dr. Kleiman's laboratory:

Shan Cen for experiments presented in Figures 3.1 and 3.3. As part of his doctoral training, the candidate also co-authored a number of articles, including:

Guo, F., Cen S , Meijuan, N , **Javanbakht, H** , Kleiman,L. 2003., *In press.* Effect of interferring RNA specific for human lysyl-tRNA synthetase upon primer tRNALys3 function and viral infectivity in HIV-1, J virology

Cen, S., A. Khorchid, **H. Javanbakht,** J. Gabor, T. Stello, K. Shiba, K. Musier-Forsyth, and L. Kleiman. 2001. Incorporation of lysyl-tRNA synthetase into human immunodeficiency virus type 1. J Virol **75**:5043-8.

Cen, S., **H. Javanbakht,** S. Kim, K. Shiba, R. Craven, A. Rein, K. Ewalt, P. Schimmel, K. Musier-Forsyth, and L. Kleiman. 2002. Retrovirus-specific packaging of aminoacyl-tRNA synthetases with cognate primer tRNAs. J Virol **76**:13111-5.

Gabor, J., S. Cen, **H Javanbakht**, M. Niu, and L. Kleiman. 2002. Effect of altering the tRNA(Lys)(3) concentration in human immunodeficiency virus type 1 upon its annealing to viral RNA, GagPol incorporation, and viral infectivity. J Virol **76**:9096-102.

Khorchid, A, **H Javanbakht**, S Wise, R Halwani, Mark A. Wainberg and L Kleiman. Sequences within $Pr160^{gag\text{-}pol}$ affecting the selective packaging of primer tRNALys3 into HIV-1. J. Mol. Biol (2000) **299**, 17-26.

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Abbreviations

AaRS	Aminoacyl-tRNA-synthetase
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
CA	Capsid HIV protein
CD4	Cluster determination antigen 4
CD8	Cluster determination antigen 8
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
DMP	Dimethyl pimelimidate
DNA	Deoxyribonucleic acid
eEF1α	Eukaryotic elongation factor 1α
EIAV	Equine infectious anemia virus
FDC	Follicular dentritic cells
FIV	Feline immunodeficiency virus
HFV	Human foamy virus
HIV	Human immunodeficiency virus
HTLV	Human T cell leukemia virus
IN	Integrase HIV protein
LysRS	Lysyl-tRNA synthetase
LTR	Long terminal repeat
MA	Matrix HIV protein

MHC	Major histocompatibility complex
MHR	Major homology region
MMTV	Mouse mammary tumor virus
MLV	Murine leukemia virus
M-PMV	Mason-Pfizer monkey virus
mRNA	Messenger RNA
NC	Nucleocapsid protein
PBS	Primer binding site
PPT	poly purine tract
PR	Protease HIV protein
RNA	Ribonucleic acid
RT	Reverse transcriptase HIV protein
RSV	Rous sarcoma virus
SIV	Simian immunodeficiency virus
SU	Surface HIV protein
SV40	Simian virus 40
TAR	Tat responsive element
TM	Transmembrane HIV protein
Vif	Virus infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X

Chapter 1

Literature review

1.1 Retroviruses

Retroviruses belong to a large family of viruses that primarily infects vertebrates. Viruses from this family reverse transcribe their genomes into DNA during their life cycle. Despite their differences, they all share similarities in their replication cycle, genome structure and virion structure. These enveloped viruses have icosahedra symmetry and are about 100 nm in diameter. The viral envelope, derived from host cell plasma membrane during budding, contains viral envelope glycoproteins. The viral core is composed of capsid protein, and contains a diploid RNA genome, viral enzymes required for replication, and host-derived tRNA, which is used as the primer for reverse transcription. The retroviral replication cycle can be divided into infective and replicative phases. The infective phase consists of viral entry, the conversion of single stranded RNA into a double stranded proviral DNA, transfer of proviral DNA into the nucleus, and integration into the genomic host. The replicative phase consists of the synthesis of viral RNA and proteins, virus assembly, budding and virus maturation.

1.1.1 Retroviruses Classification

Retroviral classification is based on their pathogenesis, morphology and type of viral assembly. Retroviruses can be divided into three subfamilies on the basis of pathologic consequence of infection (208). These are oncoviruses, lentiviruses, and spumaviruses. Oncoviruses cause malignancies. For example human T-cell leukemia virus type I (HTLV-1) can cause adult cell leukemia (177).

Lentiviruses, such as human deficiency virus type 1 (HIV-1), are "slow" viruses that cause slow progressive degenerative disease in man and animals (11, 86). Spumaviruses can induce formation of multinucleated giant cells, and can induce a cytopathic effect characterized by a vacuolized or "foamy" appearance of cytoplasm. Spumaviruses can establish persistent infections, but they have not been shown to cause tumors or any known disease in humans (198).

Retroviruses can also be classified into type A, B, C, and D, according to viral morphology and sub-cellular distribution (49, 207). Type B and type D viruses assemble immature particles in the cytoplasm prior to envelopement at the plasma membrane. In contrast, type C viruses, such as HIV-1, appear to assemble the internal structure of their particles concurrently with envelopment at the plasma membrane. Retroviruses of type B, C, D produce extracellular particles with different morphology. Mature type B particles have an electron-dense nucleoid located eccentrically in the virion. Type C particles have a centrally localized core, while the morphology of mature type D particles looks like an intermediate of type B and type C particles.

Retroviruses can also be divided into simple and complex retroviruses, according to genomic organization and regulation of gene expression (48, 49). Simple retroviruses contain gag, pol, and env genes common to all retroviruses, but do not encode regulatory or accessory proteins. Complex retroviruses contain several short open reading frames (ORFs) in addition to gag, pol, env genes. Replication cycles of complex retroviruses are more complicated than those of simple retroviruses and require other regulatory and accessory proteins.

Simple retroviruses include oncoviruses, whereas complex retrovirus consists of spumaviruses and lentiviruses. Complex retroviruses also have different patterns of gene expression. Since complex retroviruses encode regulatory proteins, their gene expression can be divided into two temporal phases, an early, regulatory phase and late, structural phase. This pattern of regulation of gene expression has not been observed in simple retroviruses.

1.2 HIV-1 Structure and Replication Cycle

HIV-1 belongs to the lentivirus genus of retroviruses. The HIV-1 is spherical and 100 nm in diameters (Figure 1). It contains 72 spikes that project outward. Each spike consists of glycoproteins made up of two components: gp120, the extracellular portion of glycoproteins that is believed to play a crucial role in binding of HIV-1 to target cells (e.g. T4 cells) and gp41, the transmembrane portion of the glycoproteins which is involved in viral cell membrane fusion. Matrix protein (p17) covers the internal surface of the membrane (5). The core is formed by the viral capsid protein p24. The core contains the two strands of viral RNA, which are associated with nucleocapsid, and other viral and cellular factors that are involved in the HIV-1 life cycle.

The HIV-1 genome is 9.7 KB in length (Figure 1.2). The proteins encoded by HIV-1 genes have been classified as: 1) structural proteins, such as matrix (MA), capsid (CA), nucleocapsid (NC, and envelope proteins; 2) the viral enzymes, protease (PR), reverse transcriptase (RT), integrase (IN); 3) the regulatory proteins Tat and Rev; 4) the accessory proteins, Vpr, Vpu, Vif and Nef.

Figure 3 shows different steps in the HIV-1 life cycle (200). CD4 is the specific receptor for the HIV-1 (129). CD4 is commonly found on T4 cells, dendritic cells, and the cells of the monocytes/macrophages lineage, thus making these cells susceptible to HIV-1 infection (166). The attachment of HIV-1 to the CD4 molecules of target cells is mediated by a specific complex formed primarily between the VI domain of the CD4 receptor and the V3 loop of gp120 (162, 195). Although CD4 is necessary and sufficient for viral attachment, its presence alone is not sufficient for viral entry (41). It has been shown that other co-receptors such as CCR5 or CXCR4 are needed for viral entry to the cells (60, 167). Those co-receptors allow fusion of the viral and cellular membranes via gp41 (35). The entry of HIV-1 into the cytoplasm of target cells occurs by a pH-independent mechanism (195).

After entry of the viral core, CA proteins dissociates, leaving a ribonucleoprotein, pre-integration complex composed of genomic RNA, primer $tRNA^{Lys3}$, RT, IN, NC, MA, and the viral accessory protein, Vpr (23). Both MA and Vpr contain nuclear import signal, which allows translocation of the complex to nucleus.

RT is an RNA-dependent, DNA dependent, DNA polymerase that converts the single stranded viral RNA genome into double–stranded DNA. RT also has RNase H activity that degrades the template genomic RNA during reverse transcription. The enzyme requires a $tRNA^{Lys3}$ primer to initiate a minus – strand strong stop DNA synthesis ((-)ssDNA). The process of reverse transcription is discussed in further detail later in this chapter (199). Once the

proviral DNA is synthesized, it is transported to the nucleus within the pre-integration complex. Inside the nucleus, proviral DNA is integrated into the host's genome by HIV-1 IN.

Transcription of the integrated proviral DNA into the host chromosome, by cellular RNA polymerase II results in three kinds of viral RNA: full-length, singly spliced, and doubly spliced transcripts. The full-length transcript codes for the Gag and Gag-Pol precursor polyproteins. The singly spliced transcripts code for Env, Vif, Vpr and Vpu. The doubly spliced transcripts code for Tat, Rev, and Nef. Prior to the synthesis of Rev, only the doubly spliced mRNA transcripts can exit the nucleus using the cellular export mechanism. Thus, Tat, Rev and Nef are the first viral proteins to be synthesized on free ribosomes in the cell cytoplasm. Following Rev synthesis, the 19Kda viral phosphoprotein moves into the nucleus via its nuclear localization signal. There it binds onto the target sequence, the Rev-response element (RRE), a 234-nucleotide RNA structure made up of 4 stem loops and a long stem (65, 70, 101, 154, 155). The RRE is within the env gene of the full length and singly spliced transcripts, 3' of the junction between gp120 and gp41 (99). A 10 amino acids leucine-rich sequence on the Rev molecules serves as the nuclear export signal (73). By interacting with cellular factors such as Crm1, Imp 2 and Ran G- protein, the unspliced and single spliced ribonucleoprotein complexes are transported to, and translocated through the nuclear pore into the cell cytoplasm for translation (50, 160). Tat is another small, regulatory protein that translocates into the nucleus after cytoplasmic synthesis. Tat acts as an essential and potent transactivator of the HIV-1 LTR. This 14 Kda

protein binds to an RNA structure called the trans-activation response element (TAR) to upregulate transcription of the viral genome.

Translation of Env mRNA takes place in the endoplasmic reticulum, where gp160 is co-expressed and complexed with CD4. Cleavage of gp160 by a cellular protease occurs in the Golgi, and glycosylation of the two cleavage products yields the mature gp120 and gp41 glycoproteins. Release of the Env glycoproteins from CD4 (assisted by Vpu) allows transport of Env to the cell surface, where it must be prevented from binding to surface CD4 (a process assisted by Nef).

The remainder of viral mRNA's are translated in the cytoplasm, and after translation, Gag and Gag-Pol are localized to the cell membrane, which is the final site for viral assembly. Assembly involves the formation of Gag complexes, which incorporate Gag-Pol, along with 2 copies of viral RNA, and certain cellular factors, such as $tRNA^{Lys}$. Budding takes place after assembly, and proteolytic processing of Gag and Gag-Pol, mediated by the viral protease, gives rise to a mature virus that is ready to infect the next cell.

1.3 Major Viral components and HIV-1 Assembly

The viral proteins are synthesized in the cytoplasm. The process of viral assembly is a complex progression of molecular associations and rearrangements whereby a core of cytoplasmic viral products interact with the cell membrane prior to budding. Gag and Gag-Pol precursors, two copies of the

viral RNA genome, and host $tRNA^{Lys}$, associate with the cell membrane and viral envelope protein, bud from the cell, and undergo protein processing to form complete, mature viral particles.

1.3.1 Proteins derived from the Gag precursor

During HIV-1 replication, the Gag precursor is synthesized. Gag alone can produce uninfectious viral-like particles (VLPs) in absence of other viral factors (197). Sometimes during or after virus budding from the cell, the HIV-1 protease cleaves Gag into the major mature Gag proteins: MA, CA, NC and p6. The protein domains in Gag are shown in figure 1.2. The proteolytic processing of Gag induces a major transformation in virion structure. MA remains associated with the inner face of the viral membrane, whereas CA condenses to form a shell around the viral RNA/NC complex. This general organization of major Gag proteins (MA, CA, and NC) is conserved among retroviruses (46). However HIV-1 Gag is also cleaved into two small spacer peptides, p1 and p2. These peptides appear to have some role in regulating the rate of cleavage by viral protease (132, 176, 209).

1.3.1.1 Matrix

The N-terminal region of Gag polyproteins contains the MA-sequences. The structure of the HIV-1 MA has been determined by both NMR and X-ray

crystallography (74). The protein folds into a compact core domain, consisting largely of α helices and a three-stranded β-sheet. In the crystal structure, MA is trimeric. MA has the ability to participate in different aspect of the life cycle. MA is important for targeting Gag polyproteins to the plasma membrane during viral assembly. In addition to targeting Gag to plasma membrane, MA is known to facilitate the viral incorporation of envelope proteins (80, 156). Moreover, MA also participates in transporting the pre-integration complex into the nucleus (24).

MA is known to direct Gag to the plasma membrane through an N-terminal myristylated glycine. It was shown that mutation of the N-terminal Gly residue blocked viral assembly and impaired binding of Gag to membrane (81, 94, 171). A basic domain between MA residues 17 and 31 has also been shown to participate in membrane binding (215, 218). This positively-charged basic domain promotes membrane binding by interacting with negatively-charged acidic phospholipids on the inner face of plasma membrane. Membrane binding and virus assembly can still be achieved if a substantial C-terminal portion of MA is deleted (85, 170).

MA appears to help incorporate Env glycoproteins into viral particles (79, 157). It was proposed that Env could incorporate into particles by interaction between sequences within the long cytoplasmic tail of gp41 and holes present in the lattice-like MA structure formed upon MA trimerization (106). In addition to the role of MA late in viral life cycle, MA can also help direct the pre-integration complex into the nucleus in the early stages of viral replication (23, 84).

1.3.1.2 Capsid

. The CA protein is the major subunit of the viral core, with about 2000 molecules per virion (89). It is 24 kda in size, and is released from the central portion of Gag by the viral protease. Structural data for CA has been obtained using X-ray crystallography (88, 165). The HIV CA contains an N-terminal domain that functions in the maturation of virus cores, and it also contains a binding site for cyclophiline A (CypA)(87). The C-terminal domain of CA contributes to Gag/Gag interaction, and contains the binding site for lysyl-tRNA synthetase (chapter 4). CA also contains the only region of Gag that displays significant homology among many different genera of retroviruses, the major homology region (MHR)(158, 210).

The C-terminus of CA is important for CA dimerization. Small deletions at the carboxy-end region of CA resulted in a reduction of virus production. In fact, viral assembly can be achieved using a minimal Gag construct composed of the Gag myristylation site, the C-terminal region of CA, a dimerization domain from a yeast transcription factor replacing NC and p6 (1). Other groups have also shown mutations within the C-terminal region of Gag prevents Gag/Gag interaction and reduce its ability to bind membrane (144). Although mutations within the N-terminal region do not prevent particle assembly, they do result in the production of viruses with reduced infectivity (18).

1.3.1.3 Nucleocapsid

NC is a nucleic acid-binding protein that has a molecular weight of 7 kda. NC has two characteristic motifs (zinc fingers motifs) made of regularly spaced cysteine and histidine residues. The retroviral Cys-His motif has the structure Cys-X2-Cys-X4-Cys-His-X4-Cys (CCHC), where most of the residues designated by Xs are not conserved either among retroviruses or between the two motifs of a single NC. The existence of these domains in NC protein is highly conserved among retroviruses except spumaviruses (161).

HIV-1 NC has many different functions. These include promoting the annealing of primer $tRNA^{Lys3}$ onto the PBS, packaging of viral RNA (182), involvement in the tight packing of Gag molecules during viral assembly (105), facilitating template switching and reducing self-annealing of the minus (-) strand DNA template during reverse transcription, and the facilitation of viral RNA dimerization (56, 183).

NC contains basic charges, which gives it the ability to bind non-specifically to RNA while, the Zinc fingers give NC its specific nucleic acid binding capacity (16). NC interaction with viral RNA occurs via NC recognition of the packaging signal, ψ, which is found on the 5' end of the HIV-1 genome (42). This signal is composed of three RNA hairpins located around the leader region (15). The first hairpin contains the so-called "kissing loop" to which NC binds, giving the protein its RNA dimerization function. Particular sequences on NC that are important for HIV-1 RNA dimerization are the basic amino acid residues that flank the first zinc finger (57), although its been suggested that both the flanking sequences and the zinc finger itself are required for dimerization in vitro (54).

$tRNA^{Lys3}$ genomic placement also requires the presence of basic amino acid residues flanking the first Zinc finger (135). NC likely unwinds the secondary structure of stem/loops in the PBS area of the genomic RNA making this region more accessible for $tRNA^{Lys3}$ (117). DNA strand exchange that occurs during reverse transcription is also facilitated by NC, where minus strand strong stop cDNA is transferred from the 5' end of the viral RNA genome to the 3' end (98). The role of NC in placement of $tRNA^{Lys3}$ is discussed in further detail later in this chapter.

The tight packing of Gag molecules that occurs during viral assembly is most likely mediated in part by NC sequences (216) within Gag. Since NC has been shown to interact with RNA in vitro, it has been proposed that the RNA molecules may act as a molecular scaffold that allows Gag molecules to pack tightly together at their carboxyl ends (40). A leucine zipper dimerization domain from a yeast transcription factor can replace the tight packing function of NC. Gag that contains a leucine zipper dimerization domain instead of NC, produces VLP with the same efficiency as the wild-type Gag (1).

1.3.1.4 p6

p6 compromise the C-terminal 51 amino acids of HIV-1 Gag. p6 mediates efficient particle release and a region of four amino acids (Pro7,Thr8, Ala9,Pro10), which is called late domain, has been implicated in this function (108). It was recently discovered that P6 late domain interacts with the Tumor

susceptibility gene 101, Tsg101 (202). One of the several functions ascribed to Tsg101 is participation in the endocytic trafficking pathway (8, 64, 140). It was shown that HIV-1 maturation and budding is dependent on Tsg101 expression (91, 159).This has indicated, for the first time that a specific component of the cellular machinery is involved in retrovirus release. p6 also contains a binding site for Vpr (131, 147).

1.3.2 Proteins derived from the Gag-Pol precursor

During translation of Gag, a minus-one ribosomal frame shift event occurs in Gag near the C-terminus of NC, with a frequency of 5-10%. This results in read-through of the Gag termination signal, and translation of the Gag-Pol precursor. During viral assembly, Gag-Pol is incorporated into Gag particles by interacting with Gag. A cytoplasmic interaction between Gag and Gag-Pol, independent of myristylation of either precursor, has been shown previously (136). However, while the incorporation of Gag-Pol into extracellular Gag particles does not depend upon myristylation of Gag-Pol, it does depend upon Gag myristylation (192). This indicates that the incorporation of Gag-Pol into viral particles does not depend upon its insertion into the plasma membrane. During the budding process, viral protease cleaves Gag-Pol into major proteins MA, CA, NC, PR, RT and IN.

1.3.2.1 Protease

HIV-1 PR is a 15 KDa aspartic acid protease (55, 173). The Gag and Gag-Pol polyproteins must be cleaved by protease, and conformational rearrangements must occur within particles, to produce mature infectious viruses. Some of these maturation events may occur simultaneously with assembly and budding (124). Protease functions as a dimer and is part of Pol. And therefore, protease activity initially depends on the concentration of Gag-Pol and the rate of autoprocessing. It has been shown that N-terminal sequences adjacent to protease effect autoprocessing (219). The p2 spacer peptide located between CA and NC may also influence the relative cleavage rates, and viral infectivity (148). Because assembly and maturation must be highly coordinated, factors that influence protease activity can have dramatic effect on viral infectivity. For instance, overexpression of protease can lead to aberrant rates of processing and decreased infectivity (148).

The crucial role of protease in the HIV-1 life cycle makes protease a prime target for drug design. Crystal structures of many protease-inhibitors have been solved (211). The active sites in PR are formed at the dimer interface, with each 99-residue monomer contributing catalytically essential aspartic acids. The active site resembles that of other aspartyl proteases, and contains the conserved triad sequence, Asp-Thr-Gly.

Several protease inhibitors are in clinical use, but mutants resistant to multiple inhibitors have been observed (45, 180). Resistance mutations are located both within the inhibitors binding pocket and at distant sites, and some mutants show increased catalytic activity (188).

1.3.2.2 Reverse Transcriptase

HIV-1 RT is a multifunctional enzyme whose activities include those of a RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase, and a ribonulcease H. During reverse transcription, the RT generates a double stranded DNA from a single stranded genomic RNA template. Mature HIV-1 RT is a heterodimer (P66/51) composed of two subunits, p66 and p51 (146) .

During production of (-)ssDNA by reverse transcription, $tRNA^{Lys3}$ is used as a primer and viral genomic RNA is used as the template for DNA polymerization. A summery of the reverse transcription process is shown in figure 1.4 (43). Synthesis of viral DNA begins with the synthesis of the (-)ssDNA. (-)ssDNA synthesis initiates from the 3' end of the primer tRNA. (-)ssDNA is the first discrete intermediate in retroviral DNA synthesis, and it is complementary to sequences from the 5' primer binding site (PBS) to the 7-methyl-G cap of the genomic RNA.

The First template switch

(-)ssDNA is then translocated from its site of synthesis at the 5' end of the genomic RNA to a position on the 3' end of the genomic RNA via complementary binding between the sense R sequence and the anti-sense R sequence in the (-)ssDNA. This process of transferring minus-strand strong-stop DNA from 5' to 3' end of the template is known as the first template switch. Viral DNA synthesis by RT is accompanied by the degradation of the RNA template by

the RNase H activity of RT, thus facilitating template switching (19). Minus-strand DNA synthesis continues until it advances past the plus strand RNA PBS sequence, and is followed by the synthesis of plus-strand viral DNA.

The initiation and synthesis of the plus stand strong stop DNA

The synthesis of plus-strand viral DNA is primed by a stretch of RNA sequences, a poly purine tract (PPT) fragment near the 3' end of the genome that remains after RNase H degradation of the genomic RNA. Continued synthesis of the plus-strand viral DNA yields the plus-strand strong-stop DNA, and this strand contains a copy of sense PBS sequence that is derived from the 3' 18 nucleotides of primer $tRNA^{Lys3}$.

The second template switch

The 3' end of the plus-strand strong-stop DNA hybridizes to the complementary sequences at the 3' end of the elongated minus-strand DNA, and this is know as the second template switch. DNA synthesis of both plus and minus strand DNA continues, and yields the full-length proviral DNA (for review see (199)).

1.3.2.3 Integrase

IN is the enzyme that is responsible for integrating the double stranded proviral DNA into the host genome. By cleaving 2-3 nucleotides from 3' terminus

of the proviral DNA, the viral IN creates recessed hydoxyl groups at one end of the genome before the nucleoprotein complex migrates into the cell nucleus. Following nuclear translocation, the IN-viral DNA complex binds to the host DNA. The 3' hydroxyl groups on the viral DNA then attack the phosphodiester bonds on the opposite strands of target DNA. The gaps are filled in via DNA synthesis, which extends the host 3' hydroxyl groups flanking the host-virus DNA junctions. Finally, ligation seals DNA breaks and the complex of integrase and proviral DNA dissociates quickly (21)

1.3.3 Env Proteins

Two viral proteins, the outer surface glycoprotein (gp120) and the trans-membrane glycoprotein (gp41) are products of HIV-1 env gene (79). The Env protein is translated from a singly spliced RNA, and synthesis of gp160, and its processing to gp120, and gp41 take place in the secretary pathway that is used for producing secreted and membrane proteins (61, 113). These env gene products play important roles during viral entry into the target cells.

Viral entry requires the binding of gp120 to the primary CD4 receptor on the cell (53, 149). The interaction between gp120 and CD4 promotes a series of conformational changes in gp120 that result in the formation or exposure of a binding site for particular members of the chemokine receptor family (CCR5 or CXCR4) that serve as co-receptors. Binding of gp120 to the co-receptor is

thought to induce additional conformational changes that lead to activation of gp41 and subsequent fusion of the viral and cellular membranes (72, 195)

There are two major classes of HIV-1: those that are macrophage (M-tropic) and non-syncytium inducing (NCI), and those that are T-cell (T-tropic) and syncytium inducing (SI). CXCR4 permits entry of T-tropic but not M-tropic viruses. CCR5 is a major co-receptor for M-tropic but not T-tropic viruses. The variable V3 loop of gp120 is an important determinant of viral tropism. However, the V3 loop is probably not the sole determinant of co-receptor specifity, because HIV-1 isolates that use the same co-receptors can have highly variable V3 sequence.

Figure 1.1. **Schematic representation of the mature HIV-1 virion.**

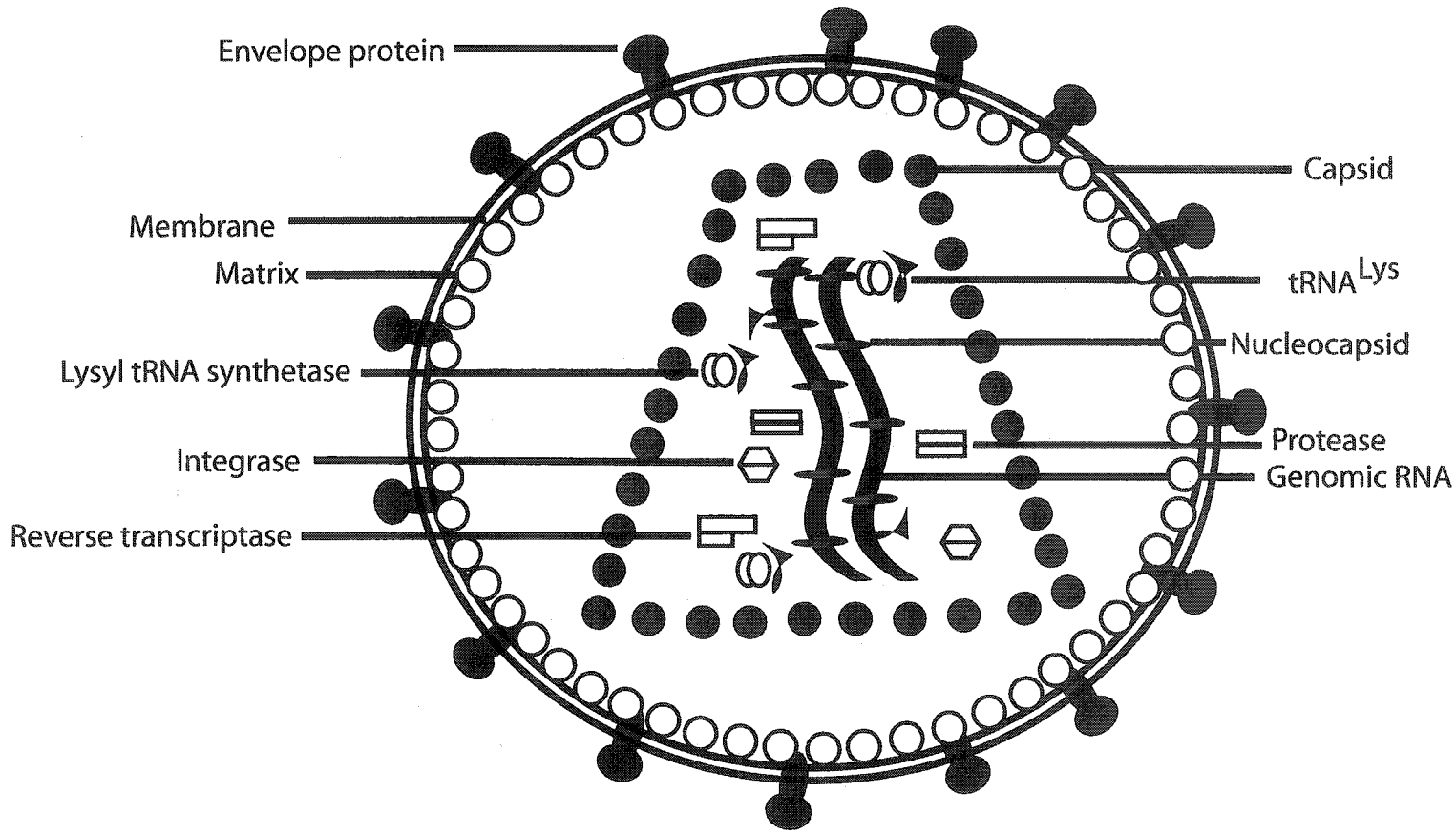
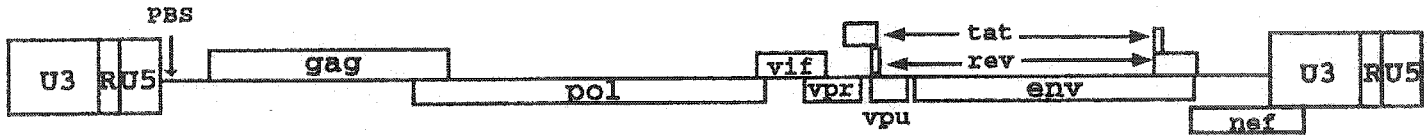
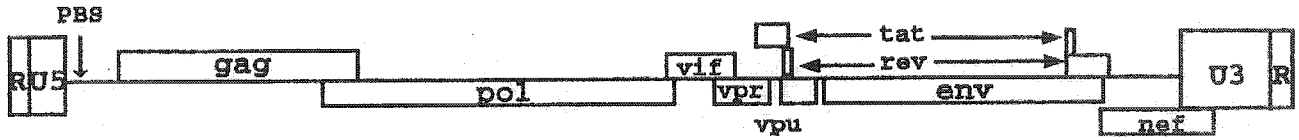

Envelope protein
Membrane
Matrix
Lysyl tRNA synthetase
Integrase
Reverse transcriptase
Capsid
tRNA Lys
Nucleocapsid
Protease
Genomic RNA

Figure 1.2. **The organization of proviral DNA, genomic RNA and structural proteins in HV-1 (not drawn in scale).**

Proviral DNA

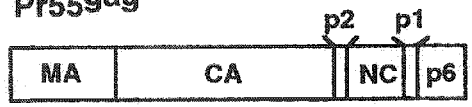


Viral Genomic RNA

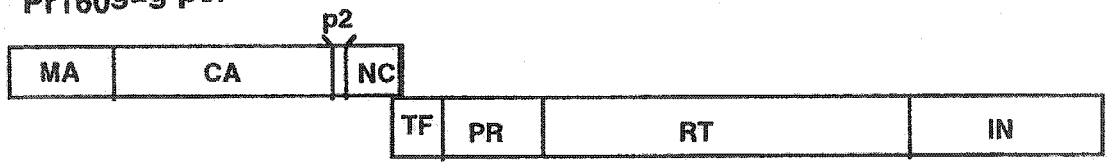


Viral Structure Proteins

$Pr55^{gag}$



$Pr160^{gag\text{-}pol}$



$gp160^{env}$

Figure 1.3. **Schematic representation of the HIV-1 life cycle.**

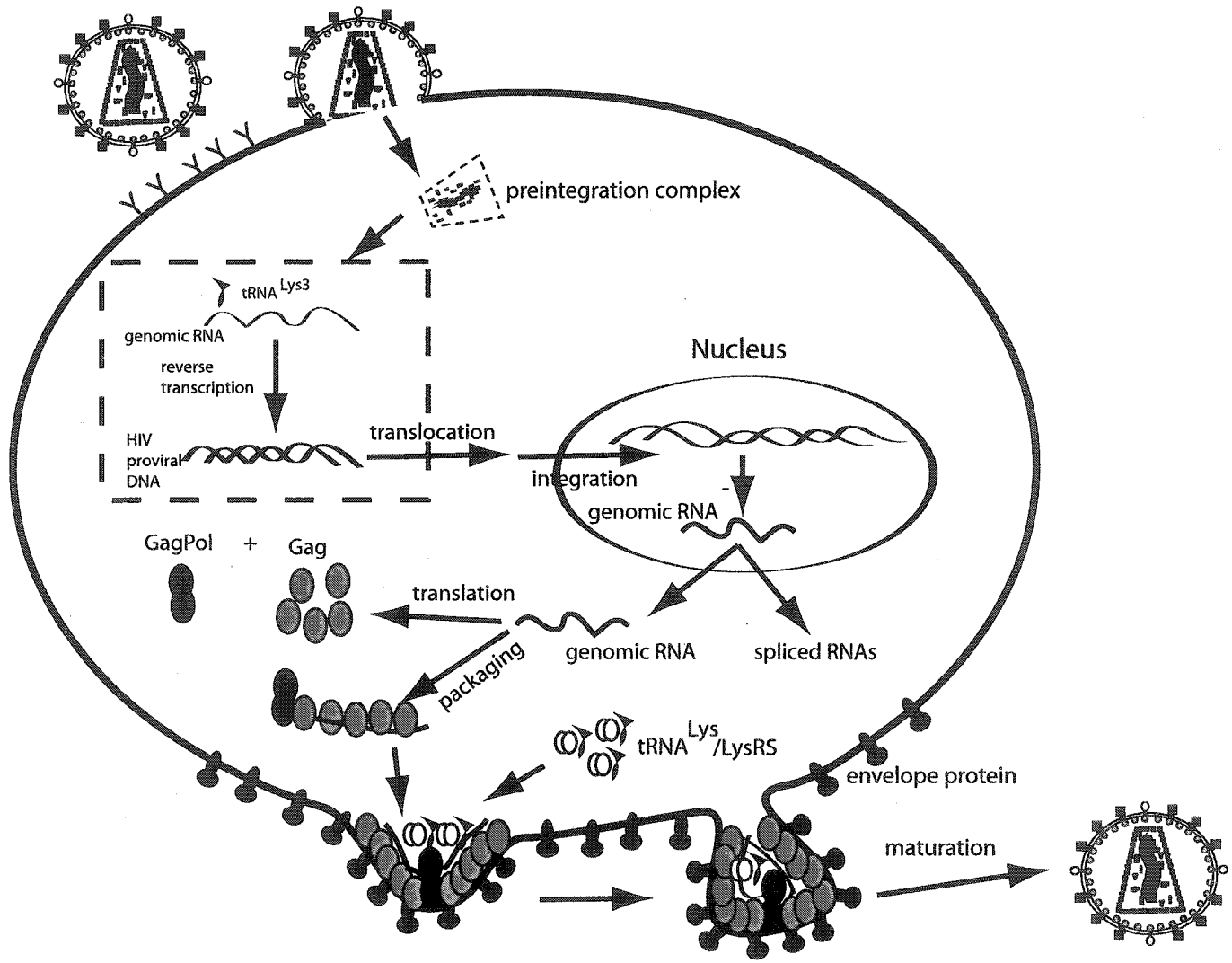
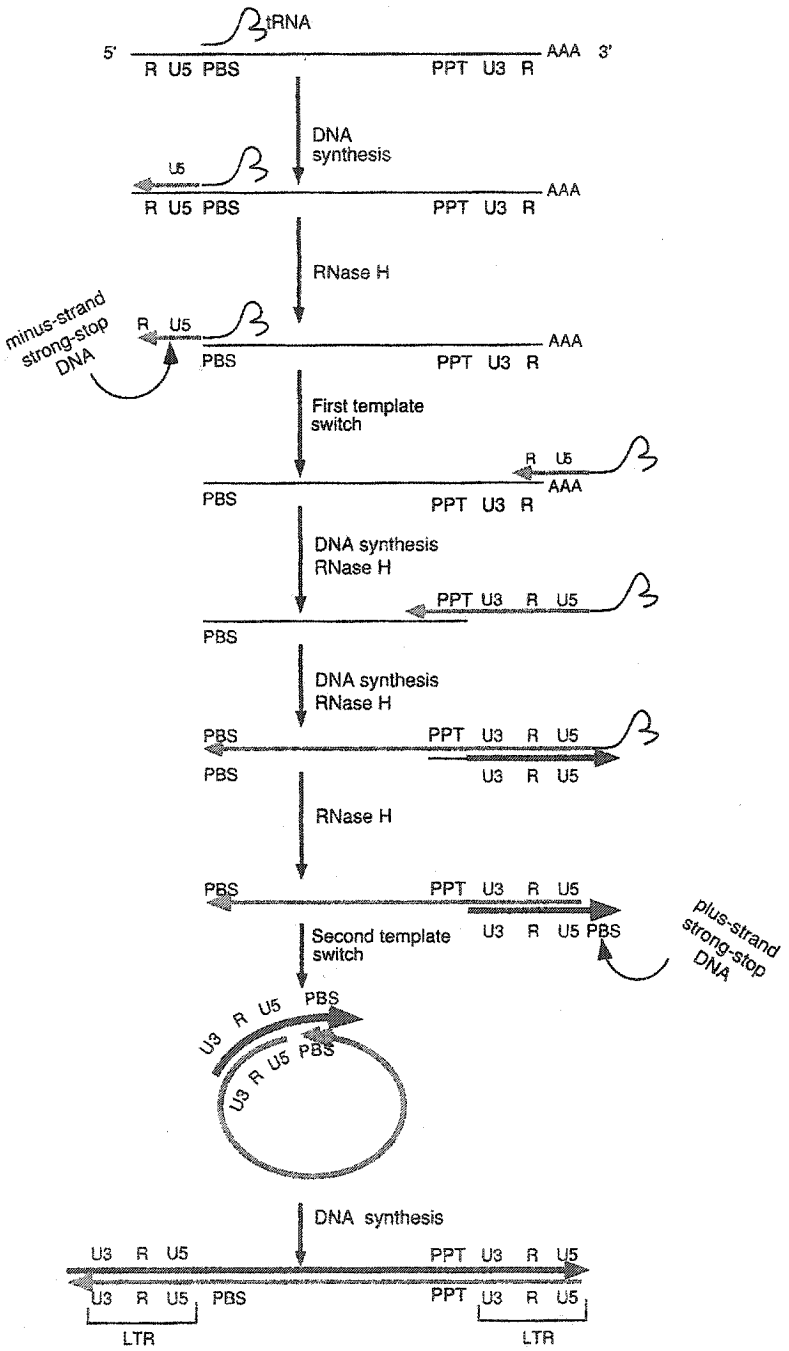

preintegration complex
tRNA Lys3
genomic RNA
reverse transcription
HIV proviral DNA
translocation
Nucleus
integration
genomic RNA
GagPol + Gag
translation
genomic RNA
spliced RNAs
packaging
tRNA Lys /LysRS
envelope protein
maturation

Figure 1.4. **A model for HIV-1 reverse transcription.**


tRNA
5'
AAA 3'
R U5 PBS
PPT U3 R
DNA synthesis
U5
AAA
R U5 PBS
PPT U3 R
RNase H
minus-strand strong-stop DNA
R U5
AAA
PBS
PPT U3 R
First template switch
R U5
AAA
PBS
PPT U3 R
DNA synthesis RNase H
PPT U3 R U5
PBS
DNA synthesis RNase H
PBS
PPT U3 R U5
PBS
U3 R U5
RNase H
PBS
PPT U3 R U5
plus-strand strong-stop DNA
Second template switch
U3 R U5 PBS PBS
U3 R U5
DNA synthesis
U3 R U5
PPT U3 R U5
U3 R U5 PBS
PPT U3 R U5
LTR
LTR

1.4 Primer tRNA in HIV-1

During retroviruses replication, the viral RNA genome is copied into a double stranded cDNA by the viral enzyme reverse transcriptase. A cellular tRNA is used to initiate reverse transcription, and is incorporated into virion during viral assembly. The primer tRNA is annealed to a 18-base sequence near the 5` end of viral RNA genome termed the primer binding site (PBS), and is used to prime a reverse transcriptase-catalyzed syntheses of minus strand cDNA, which is the first step in reverse transcription.

Different retroviral families use different tRNAs for initiation of reverse transcription. The primer tRNAs used by some retroviruses are listed in Table 1. $tRNA^{Trp}$ is the primer for many members of the avian sarcoma viruses (ASV)-avian leucosis virus (ALV) (69, 102, 175, 187, 206). $tRNA^{Pro}$ is the primer for Moloney murine leukimia virus (Mo-MuLV) . But murine retroviruses have also been reported to use alternate tRNAs as primers (44). There are three major $tRNA^{Lys}$ isoacceptors in mammalian cells (179). $tRNA^{Lys1,2}$ which represents two $tRNA^{Lys}$ isoacceptors differing by 1 bp in the anticodon stem, is the primer tRNA for several retroviruses, including Mason-Pfizer monkey virus and human foamy virus(137). $tRNA^{Lys3}$ is used as the primer for mouse mammary tumor virus (174) and the lentiviruses, such as equine infectious anemia virus, feline

immunodeficiency virus, simian immunodeficiency virus , HIV-1 and HIV-2 (137)

1.4.1 Selective packaging of Primer tRNA

Primer tRNA is selectively packaged into retroviruses during viral assembly. The term "selective packaging" refers to the increase in the percentage of the low molecular weight RNA population representing primer tRNA in going from the cytoplasm to the virus. For example, in avian myeloblastosis virus (AMV), the relative concentration of $tRNA^{Trp}$ is 1.4% in the cytoplasm but 32% in the virus (206). In HIV-1 produced from COS7 cells transfected with HIV-1 proviral DNA, both primer $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ are selectively packaged, and the relative concentration of $tRNA^{Lys}$ changes from 5-6% in the cytoplasm to 50-60% in the virus (151). In AKR murine leukemia virus (AKR-MuLV), selective packaging of primer $tRNA^{Pro}$ is less dramatic, going from a relative cytoplasmic concentration of 5-6% to 12-24% of low molecular weight RNA (206).

Both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ are packaged into HIV-1 with equal efficiency since the $tRNA^{Lys3}$: $tRNA^{Lys1,2}$ ratio in the virus reflects the cytoplasmic ratio, even when the cytoplasmic ratio is altered (110). In HIV-1, there are approximately 20 molecules of $tRNA^{Lys}$ per virion (i.e., per 2 molecules of genomic RNA) (110). Estimates of the number of each $tRNA^{Lys}$ isoacceptor

present per virion (determined using hybridization probes specific for each $tRNA^{Lys}$ isoacceptor) are 12 molecules $tRNA^{Lys1,2}$ and 8 molecules $tRNA^{Lys3}$, but these numbers must remain estimates because the viral population studied may not be homogeneous for packaging of either genomic RNA or $tRNA^{Lys}$.

1.4.2 Viral proteins involved in selective packaging of tRNA

Several studies has shown that genomic RNA packaging is not required for the incorporation of primer tRNA in ASV (178), MuLV (139), or HIV-1 (122, 151). $tRNA^{Lys}$ isoacceptors are also selectively packaged into HIV-1 independently of viral protein processing (151) . Gag particles alone do not package primer $tRNA^{Lys}$. But VLPs containing both Gag and Gag-Pol do incorporate $tRNA^{Lys}$. The RT sequences within Gag-Pol play an important role in selective packaging of primer $tRNA^{Lys}$ (151). The RT domain in HIV has been resolved into several structural subdomains termed fingers, palm, thumb, with a connection subdomain connecting the other subdomains to the C terminal RNase H subdomain (118). In vivo studies have indicated an important role of the RT thumb sub-domain within Gag-Pol in the HIV-1 packaging of $tRNA^{Lys}$. In this study deletion of Gag-Pol sequences downstream of the thumb domain did not inhibit $tRNA^{Lys3}$ incorporation into HIV-1 (128).

The RT thumb domain also seems important for the in vitro interaction between mature RT p66/p51 and $tRNA^{Lys3}$ sequences. In one study, a cyanogen

bromide fragment of RT (amino acids 230-357, containing all of the thumb sub-domain flanked by small regions from the palm and connection sub-domains) was cross-linked to a synthetic $tRNA^{Lys3}$ thiolated at base 36 in the anticodon loop (164). More recently, Dufour and colleagues cross-linked the heterodimeric p66/p51 RT to $tRNA^{Lys3}$ using cis-aquahydroxydiammino-platinum (63). After RNase A digestion of the RT-tRNA complex, a labeled oligoribonucleotide (ORN) (3` terminus of purified bovine liver $tRNA^{Lys3}$) was mainly found associated to the p66 subunit. This labeled p66-ORN complex was proteolyzed with Staphylococcus aureus V8 protease and a highly purified radioactive peptide, whose N terminal sequence corresponded to amino acid residues 241VQPI244 at the border of the thumb and the palm subdomains of the p66 subunit was obtained after two chromatographic purification steps. These in vitro experiments support in vivo findings that the thumb region of RT within Gag-Pol is important during $tRNA^{Lys}$ packaging (127).

1.4.3 Cellular Factors Involved in Selective Packaging of tRNA

tRNA is found in the cell complexed with proteins associated with translational machinery. Therefore, one of these cellular proteins could play a role in facilitating an interaction between the primer tRNA and the viral proteins. In eukaryote translation, tRNA is part of a channeled cycle (194), and aminoacyl-tRNAs are directly transferred from the aminoacyl-tRNA synthetase to the eukaryotic elongation factor 1 alpha (eEF1α) which carries tRNAs to the

ribosomes. After peptide bond formation, the deacylated tRNAs are transferred back via eEF1α to their cognate synthetases for recharging with amino acids. Two components of this translation system, lysyl-tRNA synthetase (LysRS) (32) and eEF1α (38) , have been shown to be incorporated into HIV-1.

1.4.3.1 eEF1α

eEf1α is a vital component of the translation machinery (163). In its GTP-bound form, eEF1α delivers aminoacyl-tRNA to ribosomes. Once associated with the ribosome, eEF1α hydrolyzes GTP, is released from the tRNA, and leaves the ribosome. eEF1α has also been shown to interact with HIV-1 Gag and is incorporated into the virion (39). This interaction has been shown to be RNA-dependent, and eEF1α binds to basic residues within MA and NC. eEF1α indiscriminately interacts with all aminoacyl-tRNAs. To date, there is no evidence that eEF1α plays a direct role in selective packaging of $tRNA^{Lys}$.

1.4.3.2 Lysyl-tRNA Synthetase

LysRS is a member of the aminoacyl-tRNA synthetases (aaRS) family. aaRS join amino acids with their cognate tRNAs in a high-fidelity reaction. In addition to

their role in translation, aaRSs have shown to have connections with other aspects of cellular function. These include direct participation in amino acid biosynthesis, DNA replication, RNA splicing, and aspects of eukaryotic cell biology related to cytokine function and cell cycle control (214). aaRS are divided into two main classes (I and II) based on their structural features (Table 2). Each class is further divided into three subclasses a, b and c according to their chemical properties of the substrate amino acids (114).

1.4.3.2.1 LysRS Properties

Human LysRS is the enzyme that aminoacylates $tRNA^{Lys}$, and it belongs to the class IIb aaRS family. Human LysRS has an apparent molecular weight mass of 68KDa, and like other class II aaRSs, exists as a dimer. In higher eukaryotes, LysRS is part of a high molecular weight complex aminoacyl-tRNA synthetase (HMW aaRS), with a molecular mass of 1.5 MDa, which include eight other aaRSs (Glu-ProRS, IleRS, LeuRS, MetRS, GlnRS, ArgRS, AspRS), and three auxiliary proteins: p43, p38, and p18 (214). The function of this cytoplasmic HMW aaRS complex is not known. It is possible that the $tRNA^{Lys}$/LysRS represents a transitory state, with aminoacylated tRNAs moving rapidly from these complexes, transported by eEF1α to the ribosome. LysRS domains are shown in figure 1.5. LysRS, along with other class IIb aaRS, is characterized by three specific motifs. Motif 1 has a conserved Pro residue, and is involved in

dimer formation. The motif 2 and motif 3 contain a conserved Arg residue and constitute the active site that is involved in the formation of the aminoacyl adenylate intermediates (114). Mammalian LysRS also contains a 65 amino acids N-terminal extension that functions as a nonspecific tRNA binding domain. Electrophoretic Band shift analysis showed that a N-terminally truncated hamster of LysRS displayed a 100-fold lower apparent affinity for $tRNA^{Lys}$, as compared with the native enzyme (75). LysRS also has a $tRNA^{Lys}$ anticodon-binding domain that allows it to interact specifically with $tRNA^{Lys}$.

1.4.3.2.2 LysRS Incorporation into HIV-1

LysRS is selectively packaged into HIV-1 particles (32). Interestingly, none of the other aaRSs and auxiliary proteins in the HMW aaRS were found in HIV-1 particles (Unpublished results). Moreover, our laboratory has shown that the cellular pool of LysRS that serves as the source of viral LysRS may be newly-synthesized LysRS (95). The incorporation of LysRS into viruses is very sensitive to the decrease in newly synthesized LysRS induced by small interfering RNA (siRNA) specific for LysRS. An 80% decrease in newly-synthesized LysRS is accompanied by a similar decrease in viral LysRS at a time when the total cellular LysRS has only been reduced by only 20-25% (95). LysRS in the virus has a molecular mass of 63 KDa comparing to the cytoplasmic LysRS with a

molecular weight of 68 KDa and because this truncation occurs in the absence of viral protease, a cellular protease may be involved (32).

It has been shown that HIV-1 Gag alone is sufficient to carry LysRS into Gag particles (32). Base on this fact, we have studied the Gag/LysRS interaction, using both in vitro and in vivo methods (Chapter 4). We have mapped regions in both Gag and LysRS that are required for this interaction, and conclude that the Gag/LysRS interaction depends on Gag sequences within the C-terminal domain of CA protein sequences and motif 1 of LysRS. Both regions are rich in α–helices and have been implicated in homodimerization of CA and LysRS, respectively (121). These sequences are shown in Figure 1.5.

1.4.3.2.3 Relation between LysRS and selective packaging of $tRNA^{Lys}$

The incorporation of LysRS into HIV-1 occures independent by $tRNA^{Lys3}$ packaging, since Gag VLPs that contain LysRS do not selectively package $tRNA^{Lys3}$. The additional presence of Gag-Pol is required for the selective packaging of $tRNA^{Lys}$ (150) (Figure 1.6 shows a model for this process). LysRS appears to be a limiting factor for the amount of $tRNA^{Lys}$ packaged into the virion. HIV-1 contains approximately 20 molecule of $tRNA^{Lys}$, averaging 12 molecules of $tRNA^{Lys1,2}$ and 8 molecules of $tRNA^{Lys3}$ (110). Expressing an exogenous $tRNA^{Lys3}$ gene can double the number of $tRNA^{Lys3}$ molecules in the virion, but the number of $tRNA^{Lys1,2}$ molecules/virion decreases accordingly, maintaining the same

number of total $tRNA^{Lys}$/virion (110).There is thus a factor that limits the number of $tRNA^{Lys}$ molecules packaged per virion. When cytoplasmic LysRS is increased by cotransfecting cells with an exogenous LysRS plasmid and a plasmid containing HIV-1 proviral DNA, more LysRS is packaged into the virus, and viral $tRNA^{Lys}$ doubles, with incorporation of all $tRNA^{Lys}$ isoacceptors increasing (83). This results in an increase in $tRNA^{Lys3}$ annealing to the viral RNA, and an increased in viral infectivity (82). Interestingly, the Gag:Gag-Pol ratio does not change during this process, indicating that the binding sites for the $tRNA^{Lys}$/LysRS on the Gag:Gag-Pol complexes are unsaturated. Therefore, the amount of total $tRNA^{Lys}$ incorporated into virion is directly related to the amount of LysRS package into HIV-1, which indicates that LysRS is the limiting factor for incorporation of $tRNA^{Lys}$. There are approximately 25 LysRS molecules per virion, which is similar to the number of $tRNA^{Lys}$ molecules per virion (30).

The role of LysRS in selective packaging of $tRNA^{Lys}$ was also shown by transfecting cells with HIV-1 proviral DNA and siRNA specific for LysRS. The resulting diminished cellular LysRS also resulted in viruses showing reduced $tRNA^{Lys}$ packaging, reduced $tRNA^{Lys}$ annealing to viral genomic RNA, and reduced viral infectivity (95).

In the cytoplasm, LysRS is in a complex with $tRNA^{Lys}$. LysRS primarily interacts with $tRNA^{Lys}$ anitcodon loop through its anticodon-binding domain located within the N-terminal region of LysRS. Since both of these molecules are selectively packaged into HIV-1, we investigated whether $tRNA^{Lys}$/LysRS interaction is required for selective packaging of $tRNA^{Lys}$ into HIV-1 (Chapter 2). It

was shown previously that mutations in the anticodon domain of $tRNA^{Lys3}$ inhibit interaction and, thereby, the charging of $tRNA^{Lys3}$ by LysRS *in vitro* (196). We also have shown that mutations within anticodon domain of $tRNA^{Lys3}$ inhibit *in vivo* aminoacylation for $tRNA^{Lys3}$. We have found that ability of $tRNA^{Lys3}$ to be aminoacylated is directly related to its ability to be incorporated into HIV-1(120). However, this study did not show, whether the aminoacylation reaction, in addition to the $tRNA^{Lys3}$/LysRS interaction, was required for selective packaging of $tRNA^{Lys3}$. To investigate this, we have constructed two LysRS truncated mutants. One of these, interacts with $tRNA^{Lys3}$, but has lost its ability to aminoacylate $tRNA^{Lys3}$. The other mutant LysRS has lost its ability to interact with $tRNA^{Lys3}$. These LysRS mutants were constructed by either deleting the $tRNA^{Lys3}$ binding domain within LysRS, or by deleting motif 3 that contain part of the catalytic site for LysRS (chapter 3). Both mutant LysRS species are packaged into HIV-1 with the same efficiency as the wild-type LysRS, but only motif 3–deleted LysRS results in virus containing approximately 2 times more $tRNA^{Lys3}$. These results have indicated that $tRNA^{Lys3}$/LysRS interaction, rather than aminoacylation reaction, is required for selective packaging of $tRNA^{Lys}$.

It has also been reported that the aaRS cognate to primer $tRNA^{Trp}$ in RSV, TrpRS is also selectively packaged into the virus. This indicates that aaRSs, cognate to primer tRNAs may act as the signal for targeting the primer tRNA for viral incorporation in other retroviral families (30). RSV does not package LysRS or ProRS, interestingly; ProRS was not detected in Mo-MuLV virion which uses $tRNA^{Pro}$ as a primer which might reflect the fact that Mo-MuLV shows less

selective packaging of its primer tRNA (138). Based upon this data, we have proposed a model for $tRNA^{Lys}$ packaging into HIV-1. This model is shown in figure 1.6, consists of a Gag/Gag-Pol complex interacting with a $tRNA^{Lys}$/LysRS complex, with Gag interacting with LysRS, and Gag-Pol interacting with $tRNA^{Lys}$.

1.4.4 Interaction between Primer tRNA and Genomic RNA

In retroviruses primer tRNA is found both in a free state and bound to the viral genome (175). Incorporation of primer tRNA into viruses occurs independently of genomic RNA packaging (143). Some of the proposed interactions between primer $tRNA^{Lys3}$ and HIV-1 genomic RNA are shown in figure 1.7. The 3' 18 terminal nucleotides of the primer $tRNA^{Lys3}$ are complementary to the 18 nucleotides of the PBS. Other proposed interactions are those occurring between the $tRNA^{Lys3}$ anticodon loop and an A-rich loop upstream of the PBS, and an interaction occurring between 5' portion of $tRNA^{Lys3}$ TΨC arm and viral RNA sequences upstream of the PBS (204).

Previous studies with HIV-1 have shown that the PBS sequence is not in itself sufficient to determine primer tRNA identity. Transfecting cells with HIV-1 proviral DNAs encoding PBS sequences that are complementary to tRNAs other than the natural primer tRNA yields viruses with initially slow replication kinetics (58, 142, 205). However, the mutant genomes eventually revert back to their respective wild type PBS sequences and the revertant viruses then grow at wild type rates. The reversion to the wild type PBS sequence probably results from

the reverse transcription of the 18 3' nucleotides of the natural primer tRNA which occurs during the synthesis of plus strand strong stop cDNA. After the 3' terminal nucleotides of the tRNA primer are copied, strand transfer of this strong stop DNA to the 5' terminus of the minus strand DNA results in the newly-generated PBS sequences annealing to the complementary PBS` sequences in the minus strand DNA. This model predicts that the tRNA 3` terminal sequences determine the sequence of the newly-generated PBS, and is used to explain how PBS sequences made complementary to a non-primer tRNA in retrovirus will eventually revert back to sequences complementary to a more favored natural primer tRNA. Moreover, $tRNA^{Lys3}$ remains the preferred primer tRNA in HIV-1 even when the PBS is made complementary to a tRNA with a relatively high concentration in the virus, such as $tRNA^{Lys1,2}$. Sequence comparison of the last 18 3' nucleotides of the tRNAs shows that $tRNA^{Lys1,2}$ differs from $tRNA^{Lys3}$ at 5 positions within the first 10 nucleotides, while the last 8 3' nucleotides of $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ (the first 8 of the PBS) are identical Like $tRNA^{Lys3}$, $tRNA^{Lys1,2}$ is enriched and selectively packaged into the HIV-1 viral particles (151). While the resemblance between $tRNA^{Lys1,2}$ and $tRNA^{Lys3}$ are within the suggested minimal 6 base requirement for a functional HIV-1 PBS (204), $tRNA^{Lys1,2}$ is not used as the primer for HIV-1 replication. Transfection of cells with HIV-1 proviral DNA containing $tRNA^{Lys1,2}$ complementary PBS sequence does not yield viral particles that use $tRNA^{Lys1,2}$ as a stable primer (58, 142, 205), suggesting the complementarity of the genomic RNA PBS sequence and the last 18 nucleotides of the primer tRNA is not sufficient for the template/primer interaction *in vivo*.

The reversion of altered PBSs in HIV to the natural wild type PBS suggests that there are factors other than just the PBS that influence the choice of the primer tRNA used. It has been reported that regions in the genomic RNA other than the PBS interact with the primer $tRNA^{Lys3}$. Ehresmann's group demonstrated the existence of an interaction of the USUU in the anticodon loop of $tRNA^{Lys3}$ with the A-rich loop in this U5 stem/A-rich loop (115, 117). It was shown that dethiolation of mcm^5S^2U at position 34 in the anticodon of $tRNA^{Lys3}$ destabilizes this interaction (117).

Morrow and co-workers have shown that mutations in and around the primer binding site region in HIV-1 can result in the stable use of non-$tRNA^{Lys3}$ primers such as $tRNA^{His}$ (203) and $tRNA^{Meti}$ (123) for viral replication. In the viruses using $tRNA^{His}$ as primer, $tRNA^{His}$ does not appear to be selectively packaged into the virion, but $tRNA^{Lys}$ is (217). These mutants may thus represent another example (MuLv being a natural example) in which the requirement for selective packaging of primer tRNA has been overcome. Although the use of non-$tRNA^{Lys3}$ primer tRNAs has been explained solely by RNA/RNA interactions, alterations in binding of viral or cellular proteins to the mutated genomic RNA has not been ruled out. Moreover, the efficiency with which these mutant HIV-1 virions use non-$tRNA^{Lys3}$ primers is still uncertain. In non-competitive experiments, the replication rate of mutant virions produced in the absence of wild-type HIV-1 appears to be approximately 50% of wild-type virions. But the true relative fitness of mutant vs wild-type virions, remains to be.

1.4.4.1 Primer tRNA and the Initiation of Reverse Transcription

In vitro reverse transcription, using homologous RT and genomic RNA of lentiviruses, can be resolved into initiation and elongation phases. The initiation phase is manifested by an initial buildup of 1-12 base DNA extensions of the primer tRNA (7). In the case of HIV-1, the *in vitro* extensions are 1-6 bases in length. Mn++ inhibits the elongation phase, but not the initiation phase, and the processivity of RT is less during the initiation phase than during the elongation phase. The U5 stem/loop structures may be involved in the transition between these two stages. Evidence for a discreet initiation stage of HIV-1 reverse transcription has also been found *in vivo*. In HIV-1 produced from either transfected COS7 cells or a variety of stably-infected cell lines (H9, CEM-SS, U937, and PLB), the $tRNA^{Lys3}$ annealed to the PBS appears to exist in two forms: unextended $tRNA^{Lys3}$ and $tRNA^{Lys3}$ extended by the first two nucleotides added during reverse transcription, dCMP and dTMP (112). As shown in Figure 1.7, the two-base extension would extend to the first GC base pair in the hairpin containing the A-rich loop.

Deletion of the 4 consecutive A's in the U5 stem/loop of HIV-1 does not affect $tRNA^{Lys3}$ genomic placement, either *in vitro* (7, 116) or *in vivo* (109, 145). Neither is the early pausing of reverse transcription diminished *in vitro* or *in vivo* (109) at low RT: primer/template (1:2) ratios, probably because the stem structure is still maintained. When the stem structure is destabilized, by using a 9 base extended $tRNA^{Lys3}$ as primer, pausing disappears (7). *In vivo*, HIV-1 containing a deletion of the 4 A's shows a decreased reverse transcription and

slower replication kinetics, which increase over time after the reversion of two G's to A's in the region of the stem/loop structure (145). The initiation and elongation phases of reverse transcription have been associated with low and high processivity of RT, respectively (133), and, *in vivo*, destabilization of the A-rich loop seems to result in decreased elongation due to lower RT processivity (109). The ability of HIV RT to interact with the $tRNA^{Lys3}$ anticodon bound to the A-rich loop may allow the enzyme to change conformations after a short cDNA extension, but why such an interaction is required only in HIV-1 is unknown.

Since the deletion of the A-rich loop does not alter genomic placement of primer $tRNA^{Lys3}$, it seems likely that altering the loop to interact with the new primer tRNA determined by the PBS sequences probably allows a higher processive elongation phase of reverse transcription to proceed. From this interpretation, it might be predicted that altering the PBS in a virus not containing such an anticodon-stem/loop interaction would be enough to create a new primer tRNA. This has not turned out to be the case for avian leukosis virus, where altered PBSs tend to revert to the native $tRNA^{Trp}$ PBS, and this may be due to other required interactions between $tRNA^{Trp}$ and the genomic RNA, such as the interaction shown to occur between the TΨC loop and U5 region in avian sarcoma and leukosis viruses (3, 4). Similar interaction has been recently reported between an 8-nucleotide motif in the U5 region of HIV-1 genomic RNA and TΨC loop of $tRNA^{Lys3}$ (12). This U5 region is not required for $tRNA^{Lys3}$ annealing but rather for activation of PBS-bound $tRNA^{Lys3}$ primer to initiate RT and therefore referred to as primer activation signal (PAS). The PAS element

modulates the efficiency of RT. This motif is not required for initiation of RT, since mutation of the PAS element severely impairs reverse transcription that is initiated from a tRNA primer, but not from a DNA oligonucleotide primer(13).

1.4.4.2 Major viral and cellular proteins regulating $tRNA^{Lys3}$/genomic RNA Annealing

1.4.4.2.1 Nucleocapsid protein

In HIV-1, mature NC facilities the *in vitro* annealing of $tRNA^{Lys3}$ to in vitro transcribed genomic RNA sequence (9), probably by unwinding the secondary structure of stem/loops in the PBS area of genomic RNA (117). However, the fact that nucleocapsid does not appear to denature the tRNA secondary structure is well documented (33, 34, 125).

HIV-1 NC protein contain two zinc fingers that, although important for RNA packaging (14) and the annealing step during minus strand transfer(97), are not important for $tRNA^{Lys3}$ annealing, which is more dependent upon basic amino acid regions flanking the first Cys-His box (29, 59, 103, 135). We have shown that NC protein mutations in Gag had a much greater effect on inhibiting $tRNA^{Lys3}$ annealing in vivo than the same NC mutations in Gag-Pol, indicating an important role for Gag in $tRNA^{Lys3}$ annealing. It has also been shown that Gag alone is sufficient for annealing $tRNA^{Lys3}$ to genomic RNA in vitro (71), and

processing of that Gag and Gag-Pol is not required for annealing to the PBS (31).

1.4.4.2.2 Reverse Transcriptase

There has not been clear evidence that either mature RT, or RT sequence within Gag-Pol, can facilitate primer tRNA annealing to the PBS. In ASV(174) and HIV-1 (151), the absence of RT protein and RT activity is correlated with the absence of both selective primer tRNA packaging and genomic placement of the tRNA, so it is difficult to determine if lack of primer tRNA genomic placement is due to absence of RT or to the absence of selective primer packaging. However, recent unpublished study in our laboratory indicates a role for RT sequences within Gag-Pol in the $tRNA^{Lys}$ annealing process. In this study, it was shown that C-terminal deletions of Gag-Pol, up through the RT connection domain, inhibit $tRNA^{Lys3}$ annealing to viral RNA without inhibiting selective packaging of $tRNA^{Lys}$ into the virion. Moreover, inhibition $tRNA^{Lys3}$ annealing to viral RNA can be rescued by co-transfection with a plasmid coding for Wt Gag-Pol, but not with a plasmid coding for vpr-RT. This shows that the RT connection domain sequences within precursor Gag-Pol are required for $tRNA^{Lys3}$ annealing to viral RNA, although not required for $tRNA^{Lys}$ packaging (127). Thus, these results indicate the structural requirement for RT sequences within Gag-Pol may differ for packaging of $tRNA^{Lys}$ and for its annealing to the viral RNA.

1.4.4.2.3 Lysyl-tRNA Synthetase

Viral LysRS appears to be smaller than cellular LysRS (32). Although the function of this truncation is not known, it could be required either for facilitating $tRNA^{Lys3}$ annealing (by releasing $tRNA^{Lys3}$ from the $tRNA^{Lys}$ packaging complexes), or by optimizing the initiation of RT. It has also been reported that while all detectable $tRNA^{Lys}$ in an HIV-1 infected cell is acylated, all detectable $tRNA^{Lys}$ in HIV-1 is deacylated (110), a state required for $tRNA^{Lys3}$ to function as a primer for reverse transcriptase. It is possible that truncated LysRS has role in deacylating the $tRNA^{Lys3}$ in the virus.

Table 1. Retroviruses and Their Primer tRNAs

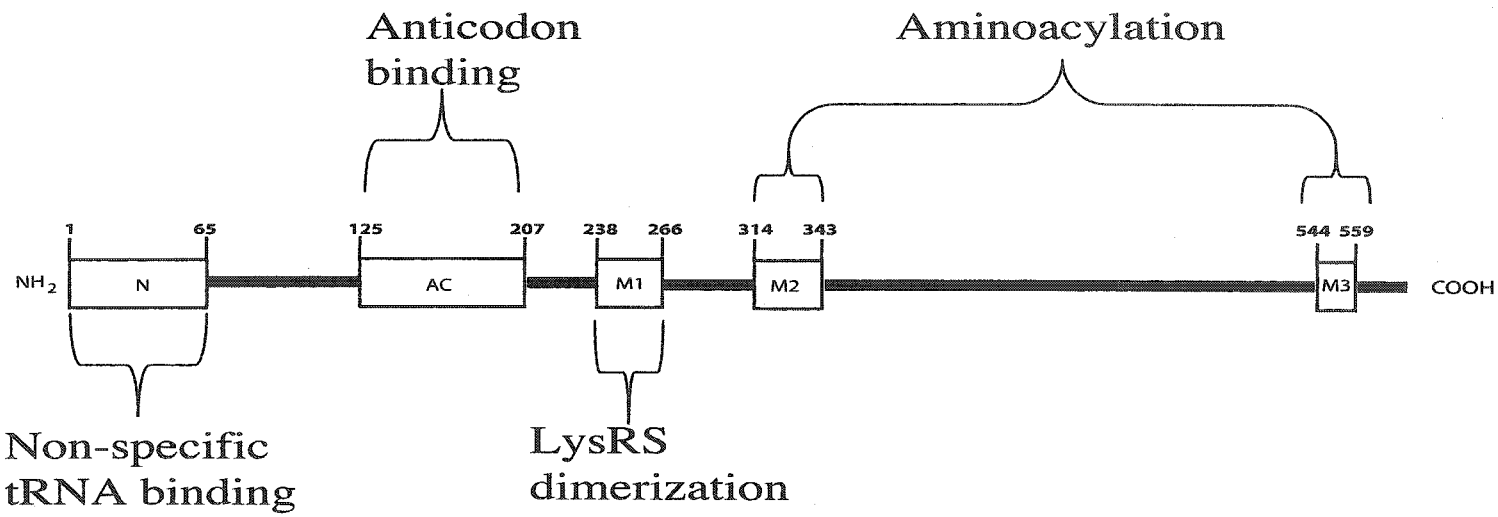
Retrovirus	Primer
Squirrel Monkey Retrovirus	$tRNA^{Lys1,2}$
Caprine Arthritis Encephalitis Virus	
Human Spumaretrovirus	
Mason-Pfizer Monkey Virus	
Simian Retrovirus 1,2(Type D)	
Visna Lentivirus	
Equine Infectious Anemia Virus	$tRNA^{Lys3}$
Feline Immunodeficiency Virus	
Human Immunodeficiency Virus 1,2	
Mouse Mammary Tumor Virus	
Simian Immunodeficiency Virus(mac)	
Baboon Endogenous Virus	$tRNA^{Pro}$
Bovine Leukemia Virus	
Feline Leukemia Virus	
Gibbon Ape Leukemia Virus	
Human T Lymphotropic Virus 1,2	
Murine Leukemia Virus	
Reticuloendotheliosis Virus	
Simian Sarcoma Virus	
Avian Sarcoma leukosis Viruses	$tRNA^{Trp}$

Table 2. **Schematic table of the two classes of tRNA synthetases, which are further divided into classes Ia, Ib, and Ic and classes IIa, IIb, IIc. The nine synthetases in higher eukaryotic cells and the three auxiliary proteins that constitute the HMW aaRS are indicated in bold**

Class I	Auxiliary proteins	Class II
Ia		IIa
IleRS **LeuRS** ValRS CysRS **MetRS** **ArgRS**	**P18** **P38** **P43**	AlaRS **ProRS** HisRS SerRS ThrRS GlyRS
Ib		IIb
GluRS **GlnRS**		AsnRS **AspRS** **LysRS**
Ic		IIc
TyrRS TrpRS		PheRS

Figure 1.5. **Summery of the domains in Lysyl-tRNA-synthtase and HIV-1 Gag. The LysRS motif1 and C-terminal of capsid are responsible for Gag/LysRS interaction.**

A. LysRS


Anticodon binding
Aminoacylation
1
65
125
207
238
266
314
343
544
559
NH2
N
AC
M1
M2
M3
COOH
Non-specific tRNA binding
LysRS dimerization

B. Gag

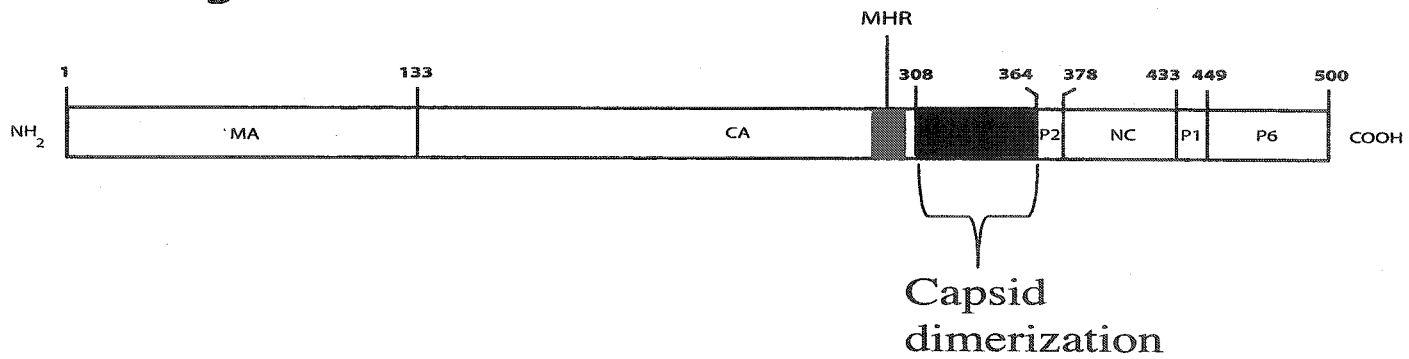

MHR
1
133
308
364
378
433
449
500
NH2
MA
CA
P2
NC
P1
P6
COOH
Capsid dimerization

Figure 1.6. **A proposed model for selective packaging of $tRNA^{Lys}$ into HIV-1. Incorporation of LysRS into HIV-1 is independent of $tRNA^{Lys3}$ packaging. Gag particles that contain LysRS do not selectively package $tRNA^{Lys3}$. However, additional presence of Gag-Pol results in selective packaging of $tRNA^{Lys}$.**

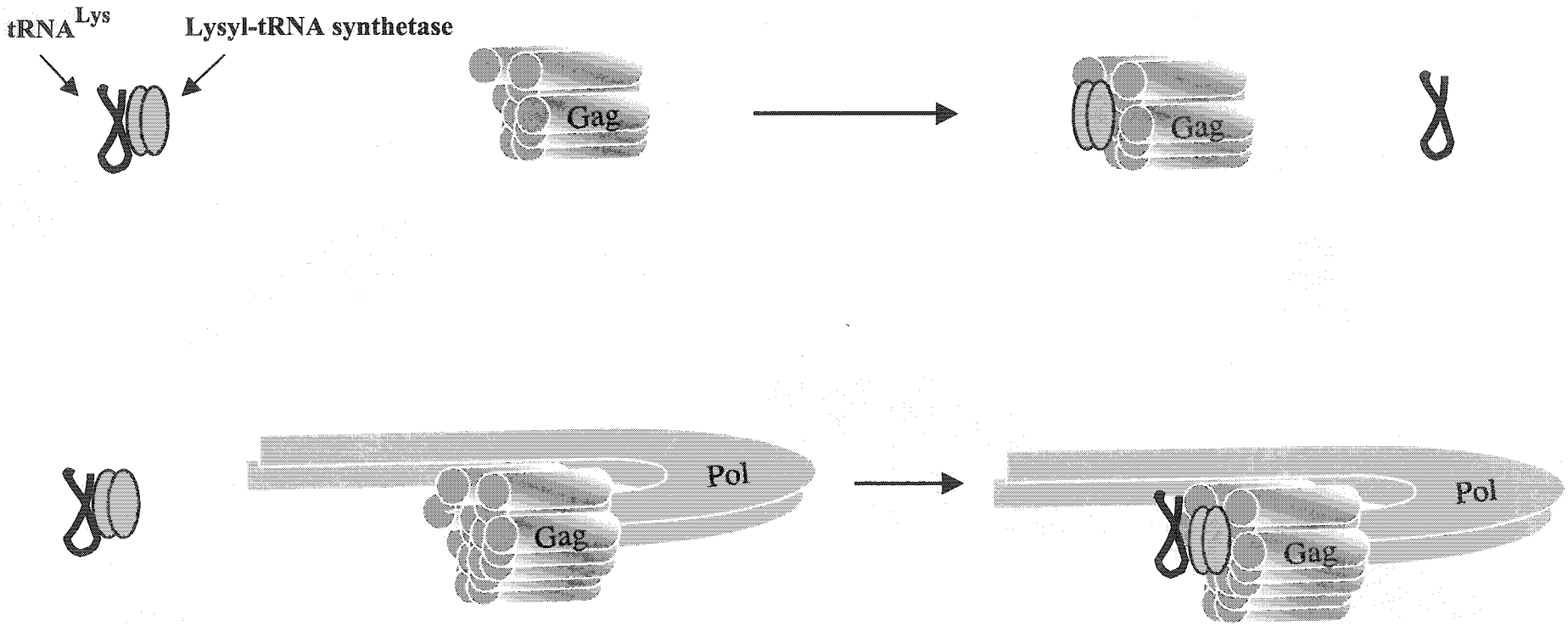
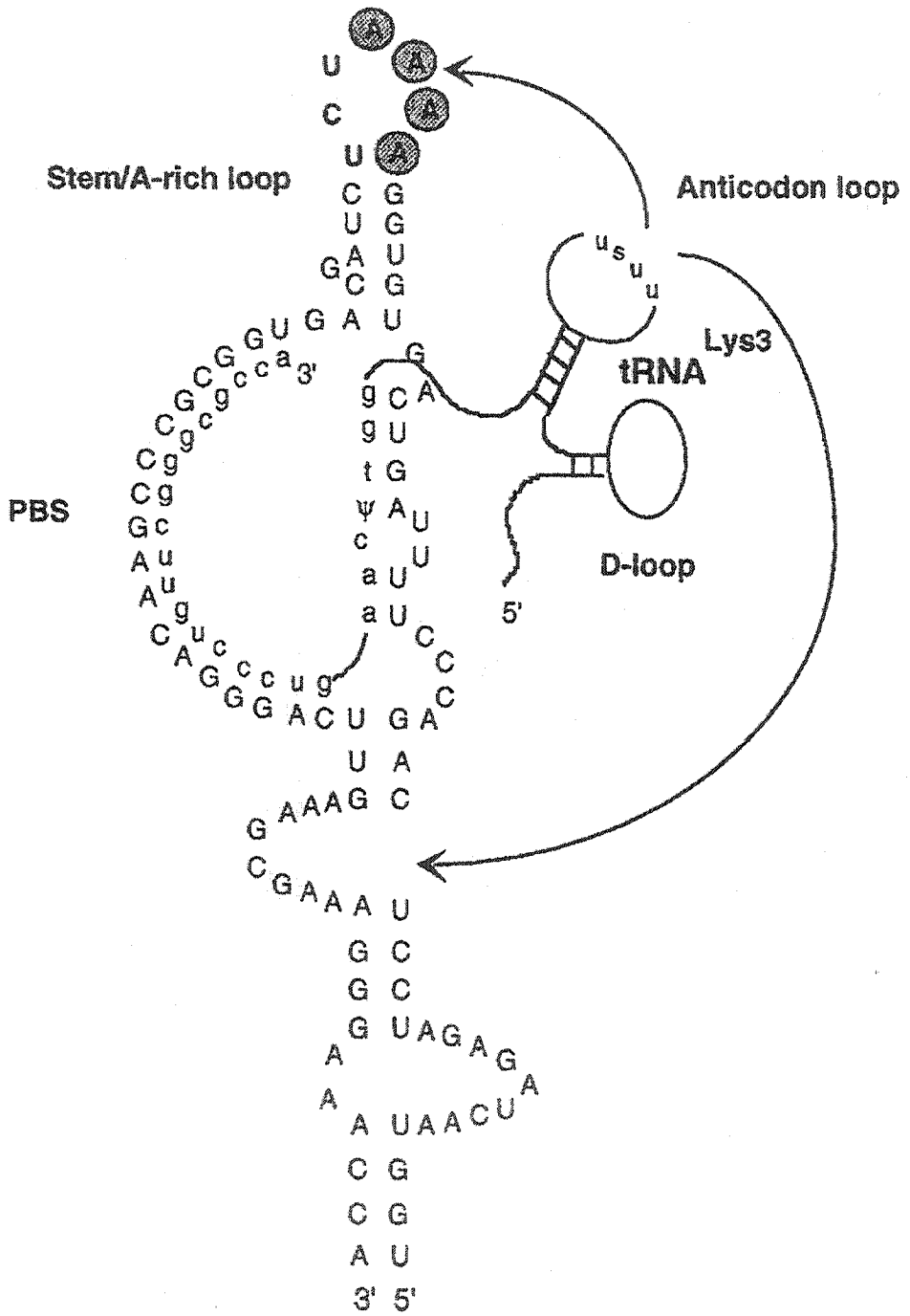

tRNA^Lys
Lysyl-tRNA synthetase
Gag
Gag
Pol
Gag
Pol
Gag

Figure 1.7.**Proposed interactions between primer $tRNA^{Lys3}$ and HIV-1 genomic RNA.**


Stem/A-rich loop
Anticodon loop
Lys3
tRNA
PBS
D-loop
5'
3'
3' 5'

Chapter 2

Correlation Between $tRNA^{Lys3}$ Aminoacylation and Its Incorporation into HIV-1

(This chapter was adapted from an article published in Journal of Biological chemistry (2002) Vol. 277, pp. 17389)

2.1 Preface

Our laboratory has shown that lysyl-tRNA-synthetase is incorporated into HIV-1. During viral assembly, in the cytoplasm, LysRS interacts with $tRNA^{Lys}$. Since both of these molecules are selectively packaged into HIV-1, we looked for evidence that this interaction is required for selective packaging of $tRNA^{Lys}$ into HIV-1. The $tRNA^{Lys}$/LysRS interaction requires an intact tRNA anticodon, and preventing mutant $tRNA^{Lys3}$ from binding to LysRS will result in reduced aminoacylation of the mutant $tRNA^{Lys3}$. For example, a previous study had shown that a mutation at position U35 within the $tRNA^{Lys3}$ anticodon had a severe effect on aminoacylation *in vitro*. Therefore, we constructed and expressed different mutant $tRNA^{Lys3}$ species, which contain a mutation at position U35 as well as at other positions within the anticodon domain. We tested the ability of these mutant $tRNA^{Lys3}$ species expressed in COS7 cells to be aminoacylated *in vivo*. We found that the mutant $tRNA^{Lys}$ which, contained mutations at positions U35 and U36, or at all three anticodon positions, remained deacylated in cytoplasm, while mutations at positions U35 alone, or at S34 and U35, were partially aminoacylated. In COS7 cells co-transfected with plasmids containing HIV-1 proviral DNA and a wild type or mutant $tRNA^{Lys3}$ gene, we found that the ability of a mutant $tRNA^{Lys3}$ to be aminoacylated in the cytoplasm was correlated with its ability to be packaged into HIV-1. This indicates the importance of the $tRNA^{Lys}$/LysRS interaction for viral incorporation of $tRNA^{Lys}$

2.2 Abstract

During HIV-1 assembly, $tRNA^{Lys}$ is selectively packaged into the virus, where $tRNA^{Lys3}$ serves as the primer for reverse transcription. Lysyl-tRNA synthetase is also selectively incorporated into HIV-1, and is therefore a strong candidate for being the signal by which viral proteins interact with $tRNA^{Lys}$ isoacceptors. Mutations in the $tRNA^{Lys3}$ anticodon have previously been shown to strongly inhibit charging of $tRNA^{Lys3}$ by lysyl-tRNA synthetase *in vitro*, and we show here that *in vivo* aminoacylation is also inhibited by anticodon changes. The order of decreasing *in vivo* aminoacylation for $tRNA^{Lys3}$ anticodon mutants is: wild-type SUU (100%) >SGU (49%) >CGU (40%) > SGA (0%) and CGA (0%). We find that the ability of these $tRNA^{Lys3}$ anticodon variants to be aminoacylated *in vivo* is directly correlated with their ability to be packaged into HIV-1. These data show that the anticodon is a major determinant for $tRNA^{Lys3}$ packaging, and support the conclusion that its productive interaction with lysyl-tRNA synthetase is important for $tRNA^{Lys3}$ incorporation into HIV-1.

2.3 Introduction

During HIV-1 assembly, the major $tRNA^{Lys}$ isoacceptors, $tRNA^{Lys1,2}$ and $tRNA^{Lys3}$, are selectively incorporated into the virion (122). $tRNA^{Lys3}$ is used as the primer tRNA for the reverse transcriptase-catalyzed synthesis of minus-strand strong stop cDNA (153). In considering the interactions between viral proteins and $tRNA^{Lys}$ during tRNA incorporation into viruses, it must be taken into account that tRNAs, like most other RNAs, are bound to proteins in the cytoplasm (194). A major tRNA-binding protein in the cytoplasm is the cognate aminoacyl-tRNA synthetase (ARS), the protein responsible for tRNA aminoacylation. Since all three $tRNA^{Lys}$ isoacceptors are packaged into HIV-1, the viral proteins might first interact with a $tRNA^{Lys}$/lysyl-tRNA synthetase (LysRS) complex during the packaging of $tRNA^{Lys}$ into the virion.

As described previously, LysRS is selectively packaged into HIV-1 (32). During HIV-1 assembly (see (197) for review), the major structural precursor protein, Gag, is assembled at the plasma membrane. Also part of the assembly complex is viral genomic RNA and another precursor protein, Gag-Pol, which contains the sequences for the viral enzymes, protease, reverse transcriptase (RT), and integrase. During or after budding of the virion from the cell, the viral protease converts these precursor proteins to the processed proteins found in

the mature virion. However, the incorporation of $tRNA^{Lys}$ occurs independently of both precursor protein processing and genomic RNA packaging (151). Gag alone is sufficient to produce extracellular Gag particles, and LysRS is packaged into these particles (32), but, the additional presence of Gag-Pol is required for $tRNA^{Lys}$ incorporation as well (151). Gag-Pol might be stabilizing the Gag /LysRS/$tRNA^{Lys}$ complex since Gag-Pol interacts with both Gag (172, 192) and $tRNA^{Lys}$ (126, 152).

The recognition and binding of ARSs with their cognate tRNAs involves interactions with the acceptor stem and, in most cases, the anticodon arms of the tRNAs (93). Based on studies of the human $tRNA^{Lys3}$/human LysRS interaction, the anticodon of $tRNA^{Lys3}$ plays an important role in LysRS recognition and binding (196). In contrast, the enzyme is relatively insensitive to mutations in the acceptor stem domain both *in vivo* (190) and *in vitro* (196). Another recent report indicated that the N-terminal domain of hamster LysRS, which is adjacent to the anticodon binding domain, while not essential for aminoacylation, improves the docking of the acceptor arm of $tRNA^{Lys3}$ into the active site of the enzyme (76).

Previous work has indicated that a certain variability in the anticodon sequence is tolerated for $tRNA^{Lys}$ packaging into virions, i.e., not only do $tRNA^{Lys3}$ (anticodon SUU, where S=mcm^5S^2U) and $tRNA^{Lys1,2}$ (anticodon CUU) appear to be packaged with equal efficiency, but a mutant $tRNA^{Lys3}$ with a CUA anticodon is packaged, although aminoacylated to only 40% of wild-type levels *in vivo*. (111). However, mutations at U35 have been shown to have a more severe effect on aminoacylation catalytic efficiency *in vitro* (196, 198), and changes at

this position have not yet been tested for their effect on $tRNA^{Lys3}$ aminoacylation in vivo, nor for packaging into virions. In this report, we have constructed different $tRNA^{Lys3}$ genes mutated at position U35 as well as at other anticodon positions, and have expressed these genes in COS7 cells also transfected with HIV-1 proviral DNA. We show that the anticodon is indeed a major determinant for $tRNA^{Lys3}$ packaging. Moreover, the ability of mutant $tRNA^{Lys3}$ molecules to be aminoacylated *in vivo* correlates directly with their ability to be packaged into HIV-1.

2.4 Experimental Procedure

2.4.1 Plasmid construction— SVC21.BH10, a simian virus 40-based vector containing wild-type HIV-1 proviral DNA, was a gift of E. Cohen, University of Montreal. SVC12.BH10Lys3 $_{UUU}$ contains the HIV-1 proviral DNA plus a wild-type $tRNA^{Lys3}$ gene with the anticodon DNA sequence TTT. SVC12.BH10Lys3$_{CGA}$, SVC12.BH10Lys3$_{CGU}$, SVC12.BH10Lys3$_{SGU}$, and SVC12.BH10Lys3$_{SGA}$ contain the HIV-1 proviral DNA plus a mutant $tRNA^{Lys3}$ gene where the anticodon DNA sequence has been changed from TTT to CGA, CGT, TGT, and TGA, respectively. Mutant $tRNA^{Lys3}$ genes were created by PCR mutagenesis (11). The amplified products were cloned into the Hpa-I site of SVC21.BH10, which is upstream of the HIV-1 proviral DNA sequence. Mutations were confirmed by DNA sequencing.

Production of wild-type and mutant HIV-1 virus— Transfection of COS7 cells with the above plasmids by the calcium phosphate method was as previously described (151). Viruses were isolated from COS7 cell culture medium 63 h posttransfection, or from the cell culture medium of infected cell lines. The virus-containing medium was first centrifuged in a Beckman GS-6R rotor at 3,000 rpm for 30 minutes and the supernatant was then filtered through a 0.2 μm filter. The viruses in the filtrate were then pelleted by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 1 h. The viral pellet was purified by centrifugation with a Beckman SW41 rotor at 26,500 rpm for 1 h through 15% sucrose onto a 65% sucrose cushion.

2.4.2 RNA isolation and analysis— Total cellular or viral RNA was extracted from cell or viral pellets by the guanidinium isothiocyanate procedure (37), and dissolved in 5mM Tris buffer, pH 7.5. Dot-blots of cellular or viral RNA were hybridized with DNA probes complementary to $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ (122), genomic RNA (29), and β -actin mRNA (DNA probe from Ambion). Two dimensional polyacrylamide gel electrophoresis (2D PAGE) of ^{32}pCp-3′-end-labeled viral RNA was carried out as previously described (122).

2.4.3 Measurement of wild-type and mutant tRNALys3using RNA-DNA hybridization— To measure the total amount of $tRNA^{Lys3}$ (combined wild-type and mutant) present in cellular or viral RNA, we used an 18-mer DNA oligonucleotide complementary to the 3′ 18 nucleotides of $tRNA^{Lys3}$ (5′ TGGCGCCCGAACAGGGAC 3′). This probe has previously been shown to hybridize specifically with $tRNA^{Lys3}$ (14), and was hybridized to dot-blots on Hybond N (Amersham) containing known amounts of purified *in vitro* transcript of $tRNA^{Lys3}$ and either cellular tRNA or viral RNA produced in cells transfected with either SVC21.BH10 alone, or SVC21.BH10 containing a wild-type or mutant $tRNA^{Lys3}$ gene. The DNA oligomer was first 5′-end-labeled using T4 polynucleotide kinase and gamma- ^{32}P-ATP (3000 Ci/mMol, Dupont Canada), and specific activities of 10^8 to 10^9 cpm/ug were generally reached. Approximately 10^7 cpm of oligomer was used per blot in hybridization reactions.

For detection of specific wild-type or mutant $tRNA^{Lys3}$, DNA probes complementary to the anticodon arm were used (see Figure 1):

wild-type $tRNA^{Lys3}_{SUU}$, (5′CCCTCAGATTAAAAGTCTGATGC3′);

$tRNA^{Lys3}_{CGA}$, (5′CCCTCAGATTTCGAGTCTGATGC-3′) ;

$tRNA^{Lys3}_{CGU}$, (5′CCCTCAGATTACGAGTCTGATGC-3′);

$tRNA^{Lys3}_{SCU}$, (5′CCCTCAGATTACAAGTCTGATGC-3′);

$tRNA^{Lys3}_{SCA}$, (5′CCCTCAGATTTCAAGTCTGATGC-3′).

In order to specifically detect the presence of $tRNA^{Lys3}$ mutants in RNA samples, blots were hybridized with 5′-^{32}P-end-labelled anticodon probes to the $tRNA^{Lys3}$ mutants in the presence of an 8-25 fold excess of non-radioactive oligonucleotide complementary to the wild-type $tRNA^{Lys3}$ anticodon arm.

2.4.4 Measurement of in vivo aminoacylation— To measure the extent of *in vivo* aminoacylation of $tRNA^{Lys3}$, the isolation of cellular or viral RNA was performed using acidic conditions required for stabilizing the aminoacyl-tRNA bond previously described (107, 201). To measure the extent of *in vivo* aminoacylation of $tRNA^{Lys3}$, the isolation of cellular or viral RNA was performed at low pH conditions required for stabilizing the aminoacyl-tRNA bond. The guanidinium isothiocyanate procedure for isolating RNA was modified by including 0.2M sodium acetate, pH 4.0 in solution D, and the phenol used was equilibrated in 0.1M sodium acetate, pH 5.0. The final isopropanol-precipitated RNA pellet was dissolved in 10 mM sodium acetate, pH 5.0, and stored at -70 ^{0}C until electrophoretic analysis. RNA was mixed with one volume loading buffer (0.1 M sodium acetate, pH 5.0, 8 M urea, 0.05% bromphenol blue, and 0.05% xylene cyanol), and electrophoresed in a 0.5mm thick polyacrylamide gel containing 8 M urea in 0.1 M sodium acetate, pH 5.0. The running buffer was 0.1 M sodium acetate, pH 5.0, and electrophoresis was carried out at 300 V for 15-

18 hours at 4^0C, in a Hoefer SE620 electrophoretic apparatus. RNA was electroblotted onto Hybond N filter paper (Amersham) using an electrophoretic transfer cell (Bio-Rad) at 750 mA for 15 min., using 1X TBE buffer (0.09M Tris-borate, pH 8.0, 2mM EDTA). Hybridization of the blots with probes for wild-type and mutant $tRNA^{Lys3}$ were performed as described above. Deacylated tRNA was produced by treating the RNA sample with 0.1 M Tris-HCl, pH 9.0 at 37^0C for 3 hours to hydrolyze the aminoacyl linkage and provide an uncharged electrophoretic marker.

2.4.5 Western blotting— Western blot analysis was performed using 300 µg of cytoplasmic or nuclear proteins, as determined by the Bradford assay (20). Cytoplasmic and nuclear extracts were resolved by SDS-PAGE followed by blotting onto nitrocellulose membranes (Gelman Sciences). Detection of the nuclear transcription factor YY1 on the Western blot utilized monoclonal antibodies to YY1 (Santa Cruz)). Western blots were analyzed by enhanced chemiluminescence (ECL kit, Amersham Life Sciences) using anti-mouse (Amersham Life Sciences) as a secondary antibody. The sizes of the detected protein bands were estimated using pre-stained high molecular weight protein markers (GIBCO/BRL).

2.4.6 Cell Fractionation— The cytoplasmic supernatant and nuclear extract were prepared from the COS7 cells as described previously (130). Western blot analysis was performed as above using anti-YY1.

2.5 Results

2.5.1 Expression of wild-type and mutant tRNALys3and their incorporation into virions— The anticodon is a major determinant for aminoacylation of human $tRNA^{Lys3}$ by human LysRS (196). The ability of $tRNA^{Lys3}$ to be aminoacylated in vitro was shown to be particularly sensitive to changes at anticodon position U35 (196). To determine whether a correlation exists between the ability of $tRNA^{Lys3}$ to be aminoacylated and incorporated into HIV-1, we have transfected COS7 cells with a plasmid containing both HIV-1 proviral DNA and a wild-type or mutant $tRNA^{Lys3}$ gene. As shown previously, this results in more $tRNA^{Lys3}$ being synthesized in the cytoplasm, and in the case of wild-type $tRNA^{Lys3}$ and $tRNA^{Lys3}$ variants examined to date, increased viral packaging has also been observed (110). However, these previously tested tRNAs did not contain changes at U35. Because the middle anticodon position is critical for aminoacylation, the different $tRNA^{Lys3}$ variants examined in this work all contained a U35G mutation; in addition to other possible anticodon mutations such as S34C or U36A (see Figure 2.1).

We have measured the ability of mutant $tRNA^{Lys3}$ to be incorporated into virions using two different hybridization probes, which are shown in Figure 2.1. We have measured changes in total $tRNA^{Lys3}$ packaged into virions (Figure 2.2) using a hybridization probe complementary to the 3′ terminal 18 nucleotides of $tRNA^{Lys3}$, which detects both wild-type and mutant $tRNA^{Lys3}$. We have also monitored the incorporation into virions of specific mutant $tRNA^{Lys3}$ using

hybridization probes complementary to the anticodon arm, i.e., probes which are specific for each mutant $tRNA^{Lys3}$ (Figure 2.4). As shown below, both types of probes yield similar conclusions, and both probes were used in Figure 6 to measure aminoacylation of either total $tRNA^{Lys3}$ or of specific wild-type or mutant $tRNA^{Lys3}$.

The data in Figure 2.2 shows both the expression of total (wild-type and mutant) $tRNA^{Lys3}$ in the cytoplasm of the transfected COS7 cells, and their incorporation into HIV-1. Figure 2.2A shows dot-blots of cellular or viral RNA hybridized with a radioactive 18 nucleotide DNA oligomer complementary to the 3′ terminal 18 nucleotides of $tRNA^{Lys3}$. The top strip represents increasing amounts of synthetic $tRNA^{Lys3}$, and the hybridization results are plotted as a standard curve in Figure 2.2B. The bottom 2 strips in Figure 2.2A show dot-blots of RNA isolated from either cell lysates containing equal amount of -actin mRNA (cell) or viral lysates containing equal amounts of viral genomic RNA (viral). Dot-blots for determining -actin mRNA and genomic RNA amounts are not shown. The relative total $tRNA^{Lys3}$/β-actin mRNA ratios are plotted in Figure 2.2 C, normalized to the value obtained in COS7 cells transfected with HIV-1 proviral DNA alone (BH10). Transfection with the wild-type $tRNA^{Lys3}$ gene or the mutant tRNALys3 genes results in an approximately two-fold increase in the cytoplasmic concentration of total $tRNA^{Lys3}$. However, as shown in Figure 2.2 D, these cytoplasmic increases in $tRNA^{Lys3}$ did not all result in increases in $tRNA^{Lys3}$ incorporation into virions. The maximum increase in $tRNA^{Lys3}$ incorporation into virions occurred with excess wild-type $tRNA^{Lys3}_{SUU}$ (1.85-fold). $tRNA^{Lys3}_{SGU}$ and

$tRNA^{Lys3}_{CGU}$ increased packaging 1.4- and 1.3-fold, respectively. $tRNA^{Lys3}_{CGA}$ showed no increase in $tRNA^{Lys3}$ incorporation, and $tRNA^{Lys3}_{SGA}$ actually showed a small decrease in packaging compared to wild-type $tRNA^{Lys3}_{SUU}$.

The changes in $tRNA^{Lys3}$ incorporation into virions upon transfection with plasmids encoding wild-type $tRNA^{Lys3}$ or anticodon variants were also visualized by 2.2D PAGE. Figure 2.3A shows the electrophoresis pattern of low molecular weight viral RNA in wild-type virions. We have previously identified spot 3 as $tRNA^{Lys3}$, and spots 1 and 2 as belonging to the $tRNA^{Lys1,2}$ isoacceptor family (122). Transfection of COS7 cells with a plasmid containing both the wild-type $tRNA^{Lys3}$ gene and HIV-1 proviral DNA results in virions containing an increase in $tRNA^{Lys3}$, and a decrease in $tRNA^{Lys1,2}$ (Figure 2.3B). Similar observations were previously reported (110). When COS7 cells are transfected with HIV-1 DNA and either $tRNA^{Lys3}_{SGU}$ or $tRNA^{Lys3}_{CGU}$, which have an intermediate ability to be packaged into virions (Figure 2.2D), the 2D PAGE patterns show an increased ratio of $tRNA^{Lys3}$ / $tRNA^{Lys1,2}$ in the virions (Figure 2.3, panels C and D). On the other hand, $tRNA^{Lys3}_{SGA}$ and $tRNA^{Lys3}_{CGA}$, which the data indicate are not packaged into virions (Figure 2.2D), do not show a significant change in the $tRNA^{Lys3}$ / $tRNA^{Lys1,2}$ ratio relative to the wild-type control (Figure 2.3, panels E and F). The $tRNA^{Lys3}$ / $tRNA^{Lys1,2}$ ratios, determined by phosphorimaging, are listed beneath each panel. Taken together, the data presented in Figures 2.2 and 2.3 show that the ability of $tRNA^{Lys3}$ anticodon variants to be packaged into HIV is directly correlated with the $tRNA^{Lys3}$ / $tRNA^{Lys1,2}$ ratio.

The experiments in Figures 2.2 and 2.3 measure total $tRNA^{Lys3}$ (wild-type and mutant) in the cytoplasm and in the virion. We have also used hybridization probes specific for each $tRNA^{Lys3}$ anticodon mutant to examine their specific expression in the cytoplasm and incorporation into virions. The dot-blots shown in Figure 2.4A measure the amount of a specific $tRNA^{Lys3}$ variant isolated from cell or viral lysates containing equal amounts of β-actin mRNA or genomic RNA, respectively. The top strip in panel A (SUU) shows the amounts of $tRNA^{Lys3}$ in cytoplasm and viruses from cells transfected with either HIV-1 alone (BH10) or transfected with HIV-1 and a wild-type $tRNA^{Lys3}$ gene (BH10Lys3). The remaining 4 strips in panel A show the amount of $tRNA^{Lys3}$ in cytoplasm and viruses from cells transfected with HIV-1 and different mutant $tRNA^{Lys3}$ genes, whose anticodon sequence is listed to the left of the strip. In each of these strips, the wild-type $tRNA^{Lys3}$ transcript was used as a control to show that the mutant anticodon probes are not detecting wild-type $tRNA^{Lys3}$. For each tRNA, a standard hybridization curve was generated and used to calculate the amount (nanograms) present in cell lysate or virus, thereby taking into account any differences in efficiencies of hybridization between the probes.

The relative $tRNA^{Lys3}$ / β-actin mRNA ratios are plotted in Figure 4B, normalized to the value found for cells transfected with HIV-1 alone (BH10). The results are very similar to that shown in Figure 2.2 using a DNA hybridization probe which measures total $tRNA^{Lys3}$. Wild type $tRNA^{Lys3}$ expression is increased significantly when cells are transfected with a wild-type $tRNA^{Lys3}$ gene. The expression of each mutant $tRNA^{Lys3}$ in the cytoplasm is similar, and results in an

approximately two- fold increase in total $tRNA^{Lys3}$ (endogenous wild-type and mutant), when probed with an oligonucleotide complementary to the 3′ end of $tRNA^{Lys3}$ (Figure 2.2C). The $tRNA^{Lys3}$ / genomic RNA ratios in virions are shown in Figure 2.4C, normalized to the value found for cells transfected with HIV-1 proviral DNA alone (BH10). The results with wild-type $tRNA^{Lys3}_{SUU}$ also match the results shown in Figure 2.2D. The incorporation of this tRNA into virions increased the $tRNA^{Lys3}$ / genomic RNA ratio to 1.87, indicating a relative incorporation of exogenous $tRNA^{Lys3}$ compared to endogenous $tRNA^{Lys3}$ of 0.87. In contrast, the relative incorporation of $tRNA^{Lys3}_{SGU}$ and $tRNA^{Lys3}_{CGU}$ was only 0.50 and 0.37, respectively, while $tRNA^{Lys3}_{CGA}$ and $tRNA^{Lys3}_{SGA}$ showed relative incorporations of 0.013 and 0.029 (Figure 2.4C).

The data in Figure 2.4 indicate that wild-type or mutant $tRNA^{Lys3}$ are expressed at approximately equal levels in the total cell lysate, but are incorporated into virions to variable extents. One explanation could be that some mutant forms of $tRNA^{Lys3}$ are not exported out of the nucleus with equal efficiency. To test this possibility, we lysed cells, and separated nuclei from cytoplasm by low speed centrifugation. A radioactive DNA probe complementary to the 3′-terminal 18 nucleotides present in all forms of $tRNA^{Lys3}$ studied here was hybridized to northern blots containing both increasing amounts of an *in vitro* $tRNA^{Lys3}$ transcript, (left side of panel A, Figure 2.5) and cytoplasmic RNA samples which contain equal amounts of β-actin mRNA (right side of panel A, Figure 2.5). The standard curve generated on the left side of panel 5A shows that the cytoplasmic $tRNA^{Lys3}$ hybridization signals obtained are within the linear

range of the standard curve. The relative total cytoplasmic $tRNA^{Lys3}$/β-actin mRNA obtained from these northern blot hybridizations is shown graphically in Figure 2.5B. These experiments indicate that both wild-type and $tRNA^{Lys3}$ variants are expressed at approximately equal amounts in the cytoplasm, consistent with the data shown in Figures 2.2 and 2.4. To ensure that effective separation of nuclear and cytoplasmic fractions was achieved in our experiments, we demonstrated that the transcription factor YY1, which concentrates in the nucleus, is only detected in the nuclear fraction (Figure 2.5C).

2.5.2 Aminoacylation of wild-type and mutant tRNALys3 in vivo— We next determined the aminoacylation state of the wild-type and mutant forms of $tRNA^{Lys3}$ examined here. The electrophoretic mobility of acylated tRNA in acid-urea PAGE is reduced relative to the deacylated form, and this property can be used to determine the extent of tRNA aminoacylation (110). Figure 6 shows northern blots of cellular and viral RNA samples electrophoresed in acid-urea gels, blotted onto Hybond N filter paper, and hybridized with radioactive DNA probes specific for $tRNA^{Lys3}$. In panel A, cellular tRNA was hybridized with the 18 nucleotide DNA oligomer complementary to the 3′-18 nucleotides of $tRNA^{Lys3}$, while in panels B-E, the cellular tRNA was hybridized with the anticodon probes specific for different $tRNA^{Lys3}$ mutants. Lane 1 in panel A shows the mobility of wild type $tRNA^{Lys3}$ deacylated in vitro at alkaline pH. As previously reported (110), in cells transfected with either the wild-type $tRNA^{Lys3}$ gene (lane 2), or not transfected with any $tRNA^{Lys3}$ gene (lane 3), the $tRNA^{Lys3}$ detected is entirely in the aminoacylated form. As also shown in panel A, a majority of the

total $tRNA^{Lys3}$ is aminoacylated in cells transfected with genes encoding $tRNA^{Lys3}_{CGU}$ (lane 5) and $tRNA^{Lys3}_{SGU}$ (lane 6). In contrast, a larger proportion of total $tRNA^{Lys3}$ is in the deacylated form in cells transfected with genes encoding $tRNA^{Lys3}_{CGA}$ (lane 4) and $tRNA^{Lys3}_{SGA}$ (lane 7).

Although the data in Figure 2.6A (lanes 4-7) suggest that the $tRNA^{Lys3}$ anticodon mutants are defective in *in vivo* aminoacylation, the total $tRNA^{Lys3}$ probed in these experiments consists of both endogenous wild-type $tRNA^{Lys3}$ and exogenous wild-type or mutant $tRNA^{Lys3}$. Thus to more directly probe the capability of the $tRNA^{Lys3}$ mutants to be aminoacylated, we used anticodon DNA probes specific to the different tRNAs (Figure 2.6, panels B-E). Lanes 8, 11, 14, and 17 represent mutant $tRNA^{Lys3}$ samples that have been deacylated *in vitro* at alkaline pH, while lanes 9, 12, 15, and 18 contain cellular RNA from cells transfected with HIV-1 proviral DNA, as well as the various tRNA genes. To demonstrate the hybridization specificity of the anticodon probes, we also probed cellular RNA from cells transfected only with HIV-1 proviral DNA (lanes 10, 13, 16, and 19). Based on the data presented in these panels, we conclude that $tRNA^{Lys3}_{SGU}$ (lane 9) and $tRNA^{Lys3}_{CGU}$ (lane 12) are aminoacylated to a significantly greater extent in vivo than $tRNA^{Lys3}_{CGA}$ (lane 15) and $tRNA^{Lys3}_{SGA}$ (lane 18). The latter two variants are present exclusively in the uncharged state. The percentage of wild-type or mutant $tRNA^{Lys3}$ present in the aminoacylated state is shown graphically in panel F.

2.6 Discussion

Figure 2.7 shows a plot of the relative $tRNA^{Lys3}$ incorporation into HIV-1 for each of the anticodon variants tested in this work *versus* the percentage aminoacylated tRNA detected *in vivo*. The linear correlation is striking, and shows that the ability of $tRNA^{Lys3}$ to be incorporated into HIV-1 is closely correlated with its ability to be aminoacylated. The efficiency of aminoacylation by ARSs may be described by an overall specificity constant (k_{cat}/K_M), which contains a catalytic rate constant (k_{cat}), as well as a binding parameter (K_M). Previous studies indicated that the U35G anticodon mutation in human $tRNA^{Lys3}$ abolished *in vitro* aminoacylation (>3000-fold decrease in k_{cat}/K_M), and that this change affected both LysRS binding and catalysis (196). Despite this dramatic effect on the ability to be aminoacylated with lysine *in vitro*, we report here that mutants with changes only in U35 ($tRNA^{Lys3}_{SGU}$) or in both S34 and U35 ($tRNA^{Lys3}_{CGU}$) are still aminoacylated *in vivo* to significant levels (Figure 2.6). These mutations change the anticodon to the sequence found in $tRNA^{Thr}$. The anticodon is a major recognition element for *E. coli* threonyl-tRNA synthetase *in vitro* (104). Moreover, a change in the anticodon can switch the identity of *E. coli* elongator methionine tRNA ($tRNA^{Metm}$) from methionine to threonine, showing that it is also a major identity determinant *in vivo* (189). Assuming human threonyl-tRNA synthetase maintains this strong anticodon recognition, it may be that the XGU anticodon variants tested here are at least partially charged with threonine *in vivo*.

Based on *in vitro* studies, a single U36A change in human $tRNA^{Lys3}$ affects primarily the k_{cat} parameter, with only a 3-fold increase in K_M and an overall 182-fold decrease in catalytic efficiency. Coupling the U36A mutation with a U35G change abolished *in vivo* aminoacylation and packaging into HIV-1 (Figure 2.7). Both the UGA and the CGA anticodons correspond to sequences normally found in $tRNA^{Ser}$ isoacceptors. It has been shown that human seryl-tRNA synthetase does not use the anticodon as a recognition element, but instead requires a long variable extra arm (2, 213). Thus, it is unlikely that human $tRNA^{Lys3}_{SGA}$ or $tRNA^{Lys3}_{CGA}$ would be aminoacylated by serine *in vivo*, consistent with our observations that *in vivo* aminoacylation is abolished in these variants.

In this work, the data indicate that the anticodon of human $tRNA^{Lys3}$ is a major determinant for packaging into HIV-1 (Figure 2.7), with the caveat that we do not yet know if altering the anticodon sequence affects modifications elsewhere in the tRNA molecule. However, a clear correlation between aminoacylation and packaging has been established (Figure 2.7). Based on this data, we cannot determine whether aminoacylation itself is a prerequisite for packaging, or whether the major factor is LysRS binding. To address this question, the identity of the amino acid attached to the mutant tRNAs that are incorporated to significant extents (i.e. $tRNA^{Lys3}_{SGU}$ and $tRNA^{Lys3}_{CGU}$) must be determined, and these experiments are planned.

The data presented in this work supports a model in which the $tRNA^{Lys3}$/LysRS interaction is important for $tRNA^{Lys3}$ incorporation into viruses. We also show that the anticodon is a major determinant for packaging. However,

the anticodon sequence has also been shown to contribute to the *in vitro* binding of mature reverse transcriptase in studies using either native $tRNA^{Lys3}$ (186) or unmodified $tRNA^{Lys3}$ transcripts (10, 212). Other data suggest that RT sequences in the precursor Gag-Pol polyprotein interact with $tRNA^{Lys3}$ during its incorporation into virions (126, 151). Thus, an anticodon mutation might also weaken this $tRNA^{Lys3}$/Gag-Pol interaction. However, there is no clear evidence to date that the interaction between RT sequences in Gag-Pol and the anticodon of $tRNA^{Lys3}$ is involved in its packaging into HIV-1, and, in fact, mutations in RT that prevent the enzyme from interacting with the $tRNA^{Lys3}$ anticodon *in vitro* (6) were reported to have no effect on viral packaging of $tRNA^{Lys3}$ (126).

Figure 2.1. **Sequence of human $tRNA^{Lys3}$ folded into the cloverleaf secondary structure.** The arrows point to the anticodon mutations created in this work, and the mutant tRNA species are listed as well. Sequences complementary to the DNA hybridization probes are indicated by the solid lines. The probe complementary to the 3´-18 nucleotides will hybridize with both wild-type and $tRNA^{Lys3}$ anticodon variants, while the probes complementary to the anticodon arm are specific for each mutant $tRNA^{Lys3}$.

$tRNA^{Lys3}$ Mutants

$tRNA^{Lys3}CGA$

$tRNA^{Lys3}CGU$

$tRNA^{Lys3}SGU$

$tRNA^{Lys3}SGA$

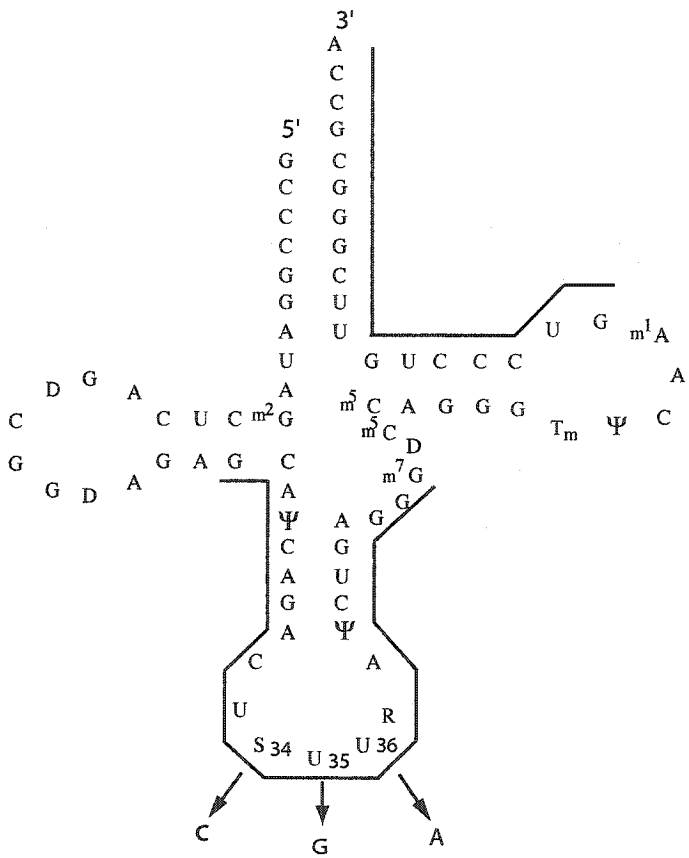


Figure 2.2. **Expression of total $tRNA^{Lys3}$ in cells and viruses.** COS7 cells were transfected with a plasmid containing HIV-1 proviral DNA and a wild-type or mutant $tRNA^{Lys3}$ gene. Dot-blots of cellular or viral RNA, containing equal amount of either β-actin mRNA (cellular RNA) or genomic RNA (viral RNA) were hybridized with a DNA probe complementary to the 3´- terminal 18 nucleotides of $tRNA^{Lys3}$ to determine the total amount of $tRNA^{Lys3}$ present (A,B). The top strip in (A) is a dot-blot of increasing amounts of an *in vitro* $tRNA^{Lys3}$ transcript, used to generate the standard curve shown in panel (B). The bottom two strips in panel (A) show dot-blots of cellular or viral RNA isolated from cells transfected with HIV-1 proviral DNA and a wild-type or mutant $tRNA^{Lys3}$ gene. 1, cells transfected with HIV-1 DNA alone (BH10, also referred to as "none" in panels C and D). 2-6 represents cells transfected with with HIV-1 DNA and $tRNA^{Lys3}$ genes coding for the following anticodon sequences: 2, SUU (wild-type); 3, CGA; 4, CGU; 5, SGU; 6, SGA. The normalized results are plotted in panels C and D for cellular or viral RNA blots, respectively.

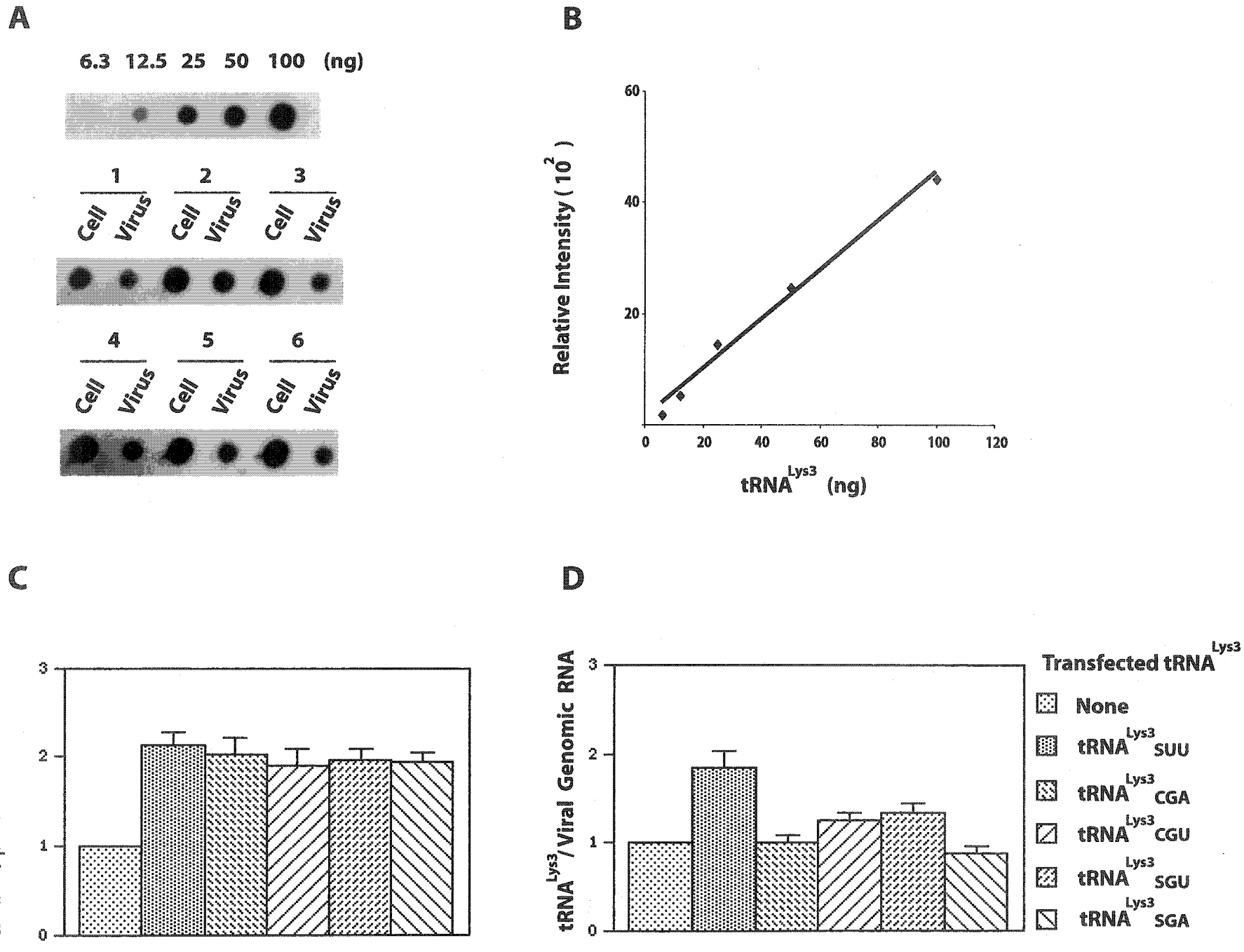

A
6.3 12.5 25 50 100 (ng)
1 2 3
Cell Virus Cell Virus Cell Virus
4 5 6
Cell Virus Cell Virus Cell Virus
B
Relative Intensity (10^2)
60
40
20
0 20 40 60 80 100 120
tRNA^Lys3 (ng)
C
tRNA^Lys3 / β-actin mRNA
3
2
1
0
D
tRNA^Lys3 / Viral Genomic RNA
3
2
1
0
Transfected tRNA^Lys3
None
tRNA^Lys3 SUU
tRNA^Lys3 CGA
tRNA^Lys3 CGU
tRNA^Lys3 SGU
tRNA^Lys3 SGA

Figure 2.3. **Two dimensional PAGE patterns of viral low molecular weight RNA.** RNA was extracted from virions produced from COS7 cells transfected with a plasmid containing HIV-1 proviral DNA and a wild-type or mutant $tRNA^{Lys3}$ gene. The RNA was 3´-end-labeled *in vitro* with ^{32}pCp, and analyzed by 2D PAGE. A, cells transfected with HIV-1 DNA alone, i.e., containing only endogenous $tRNA^{Lys3}$ (Endogenous). B-F represent cells transfected with with HIV-1 DNA and $tRNA^{Lys3}$ genes containing the anticodon sequences listed above each panel. The numbers under each panel correspond to the $tRNA^{Lys3}$ / $tRNA^{Lys1,2}$ ratios, and were determined by phosphorimaging.

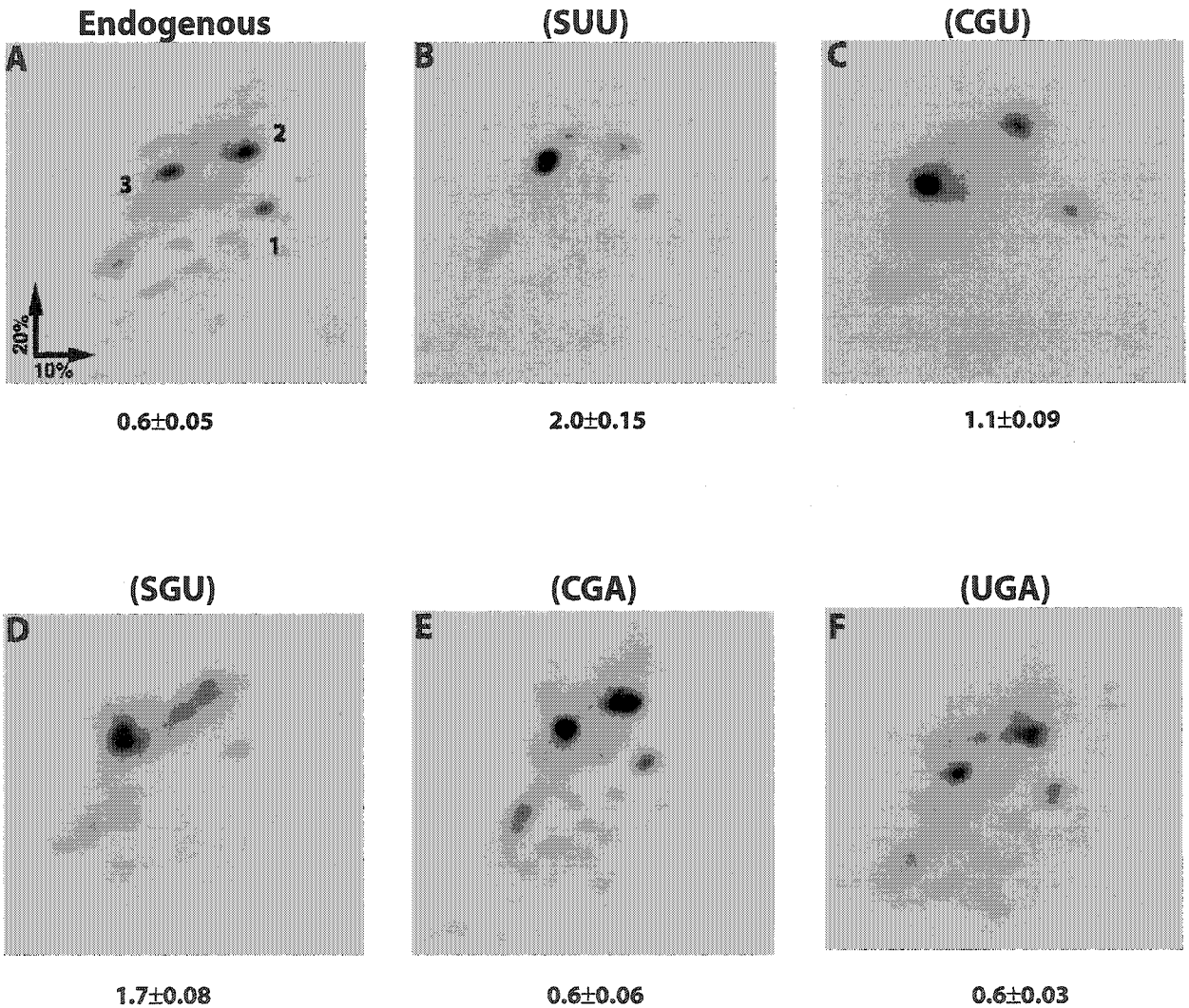

Endogenous
(SUU)
(CGU)
A
B
C
2
3
1
20%
10%
0.6±0.05
2.0±0.15
1.1±0.09
(SGU)
(CGA)
(UGA)
D
E
F
1.7±0.08
0.6±0.06
0.6±0.03

Figure 2.4. **Expression of specific wild-type and mutant tRNA^{Lys3} in cells and viruses.** COS7 cells were transfected with a plasmid containing HIV-1 proviral DNA and a wild-type or mutant tRNA^{Lys3} gene. For each strip in panel A, the left portion contains dot-blots of increasing amounts of an *in vitro* wild-type or mutant tRNA^{Lys3} transcript, used to determine differences in efficiencies of hybridization for different anticodon probes. The right portion contains dot-blots of cellular or viral RNA, containing equal amount of either β-actin mRNA (cellular RNA) or genomic RNA (viral RNA), which were hybridized with a DNA probe complementary to anticodon arm of each wild-type and mutant tRNA^{Lys3}, as shown in Figure 2.1. The control in the bottom 4 strips is the wild-type tRNA^{Lys3} *in vitro* transcript, and shows that the anticodon probes do not detect wild-type tRNA^{Lys3}. The letters to the left of the strips represent the anticodon sequence of the tRNA^{Lys3} detected. In the SUU strip, cells were transfected with HIV-1 DNA alone (BH10, also labeled as "none" in panels B and C), or with HIV-1 DNA plus a wild-type tRNA^{Lys3} gene, and probed with a DNA probe complementary to anticodon arm of wild-type tRNA^{Lys3}. The normalized results are plotted in panel B (cellular) and in panel C (viral).

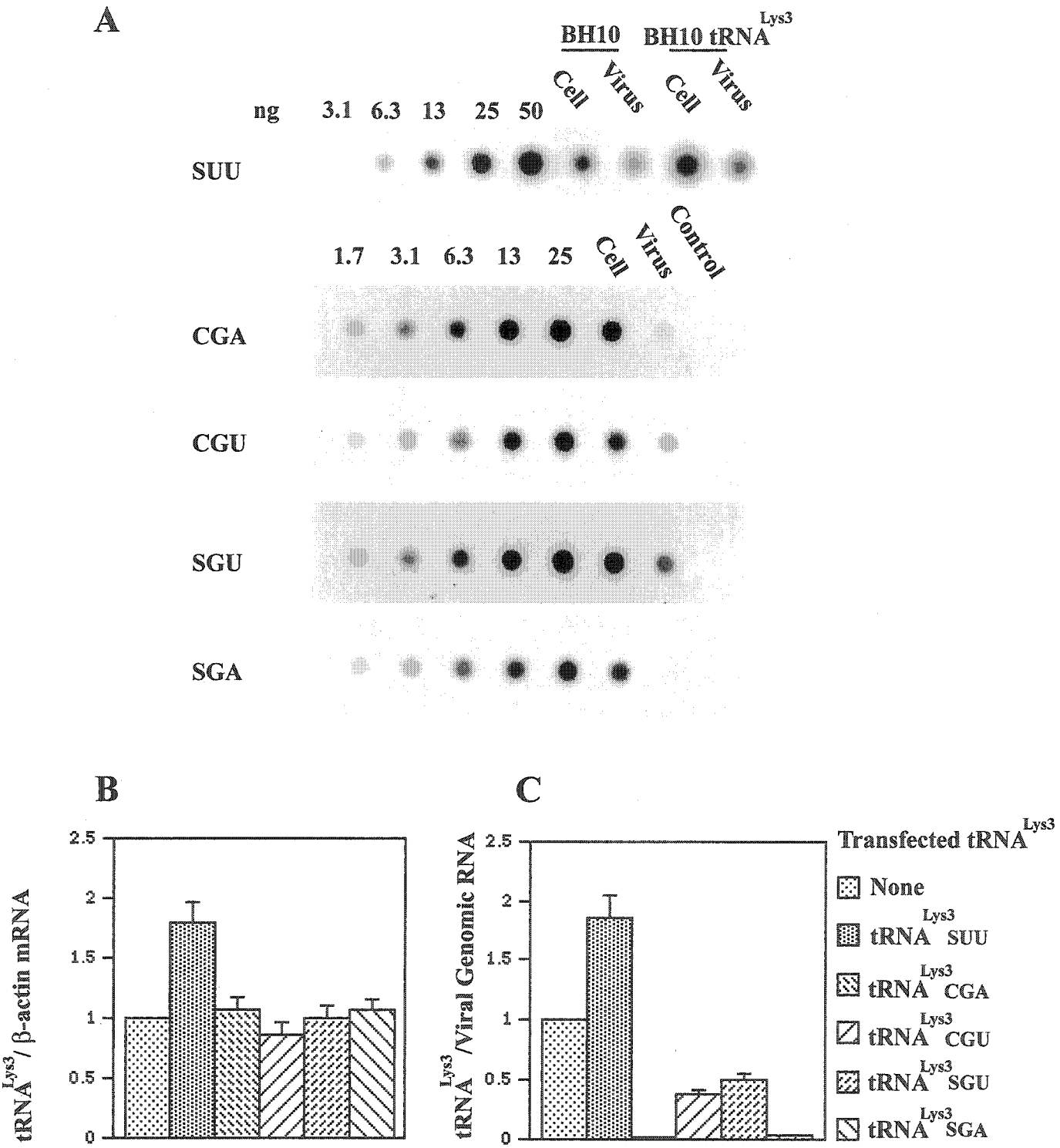
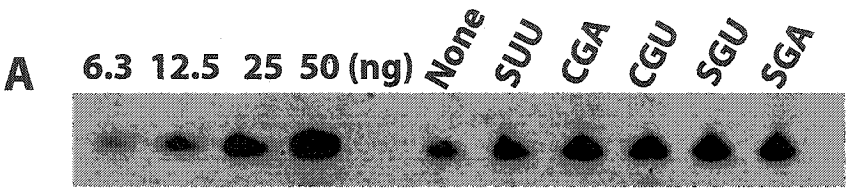
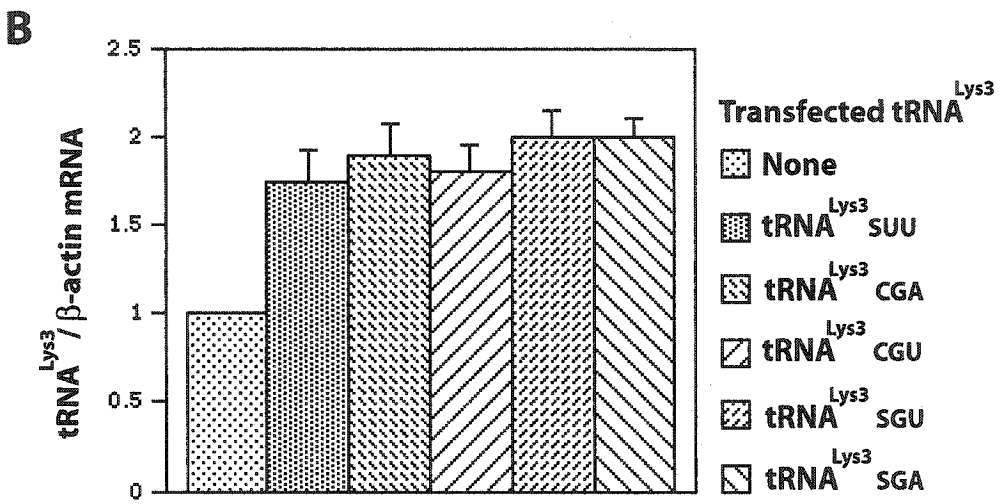

A
BH10
BH10 tRNA Lys3
Cell
Virus
Cell
Virus
ng
3.1
6.3
13
25
50
SUU
1.7
3.1
6.3
13
25
Cell
Virus
Control
CGA
CGU
SGU
SGA
B
tRNA Lys3 / β-actin mRNA
2.5
2
1.5
1
0.5
0
C
tRNA Lys3 /Viral Genomic RNA
2.5
2
1.5
1
0.5
0
Transfected tRNA Lys3
None
tRNA Lys3 SUU
tRNA Lys3 CGA
tRNA Lys3 CGU
tRNA Lys3 SGU
tRNA Lys3 SGA

Figure 2.5. **Cytoplasmic expression of wild-type and mutant $tRNA^{Lys3}$.** COS7 cells were transfected with a plasmid containing HIV-1 proviral DNA and a wild-type or mutant $tRNA^{Lys3}$ gene, and differential centrifugation was used to separate nuclei and cytoplasm. A: A radioactive DNA probe complementary to the 3′-terminal 18 nucleotides present in all forms of $tRNA^{Lys3}$ studied here was hybridized to northern blots containing both increasing amounts of an *in vitro* $tRNA^{Lys3}$ transcript, (left side of panel A) or cytoplasmic RNA samples which contain equal amounts of β-actin mRNA (right side of panel A). On the right side of panel A, "none " refers to the absence of transfection of a gene coding for $tRNA^{Lys3}$, while the wild-type or mutant $tRNA^{Lys3}$ anticodon of transfected $tRNA^{Lys3}$-encoding genes is listed above each of the remaining lanes. B: Graphic representation of the total cytoplasmic $tRNA^{Lys3}$/β-actin mRNA obtained from the data in panel A, and normalized to the value obtained in HIV-1 transfected cells not transfected with a $tRNA^{Lys3}$-encoding gene. C: Western blot of nuclear and cytoplasmic fractions of transfected cells, labeled as in panel A. Blots probed with antibody to YY1, a nuclear transcription factor. N, nuclear fraction; C, cytoplasmic fraction.


A
6.3 12.5 25 50 (ng)
None
SUU
CGA
CGU
SGU
SGA


B
2.5
2
1.5
1
0.5
0
tRNA Lys3 / β-actin mRNA
Transfected tRNA Lys3
None
tRNA Lys3 SUU
tRNA Lys3 CGA
tRNA Lys3 CGU
tRNA Lys3 SGU
tRNA Lys3 SGA

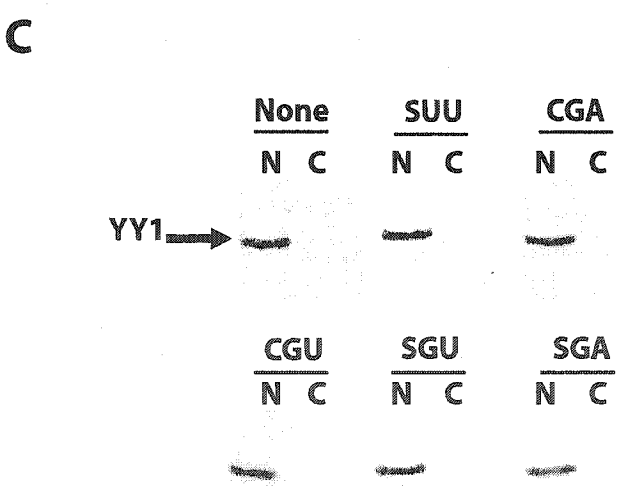
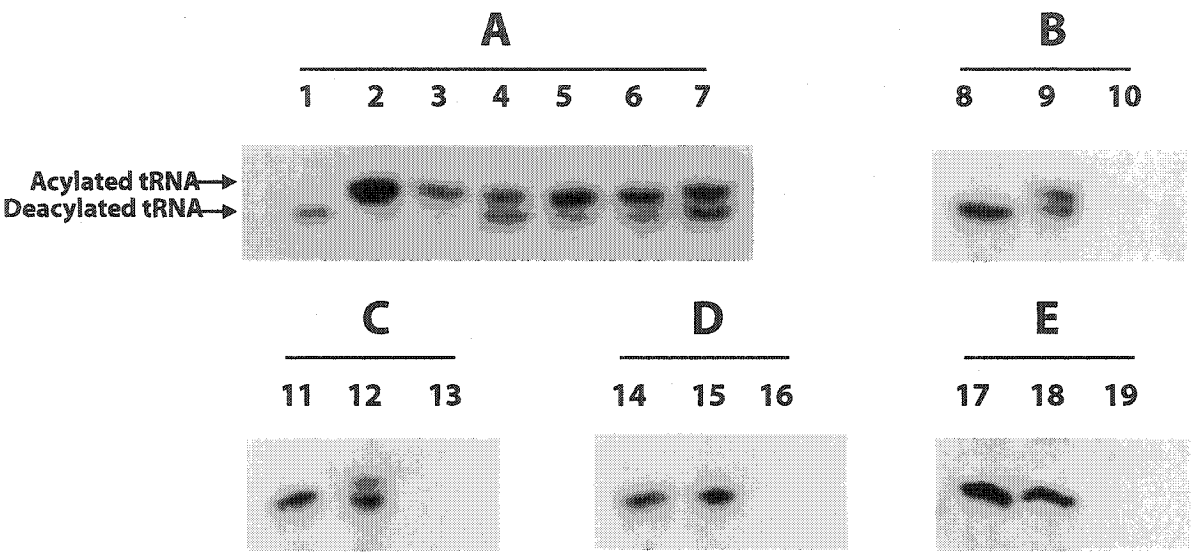

C
None
SUU
CGA
N C
N C
N C
YY1
CGU
SGU
SGA
N C
N C
N C

Figure 2.6. **Electrophoretic detection of acylated and deacylated $tRNA^{Lys3}$.** Cellular RNA was isolated and fractions containing equal concentrations of β-actin mRNA were electrophoresed under acidic conditions as described in the text. Northern blots of the cellular RNA were hybridized with either the 3′-terminal DNA probe, which hybridizes to all forms of $tRNA^{Lys3}$ (A), or with anticodon probes specific for each mutant $tRNA^{Lys3}$ (B-E). The first lane in each panel (1, 8, 11, 14, 17) represents cellular RNA, which was first exposed to alkaline pH to deacylate the tRNA (see Experimental Procedures). In panel A: 3, cells transfected with HIV-1 DNA alone (BH10, also referred to as "none" in panel F). 2, and 4-7, cells transfected with HIV-1 DNA and $tRNA^{Lys3}$ genes coding for the following tRNA anticodon sequence: 2, SUU; 4, SGA; 5, SGU; 6, CGU; 7, CGA. In panels B-E: The middle lane (9, 12, 15, 18) represents the RNA from cells transfected with HIV-1 DNA and $tRNA^{Lys3}$ genes coding for the following tRNA anticodon sequence: B, SGA; C, SGU; D, CGU; E, CGA. The last lane in each of these panels (10, 13, 16, 19) represents RNA extracted from cells transfected only with HIV-1 proviral DNA. The percentage of aminoacylation, as determined from lanes 2 and 3 in panel A and the middle lanes in panels B-E, is shown in panel F.


A
1 2 3 4 5 6 7
B
8 9 10
Acylated tRNA
Deacylated tRNA
C
11 12 13
D
14 15 16
E
17 18 19

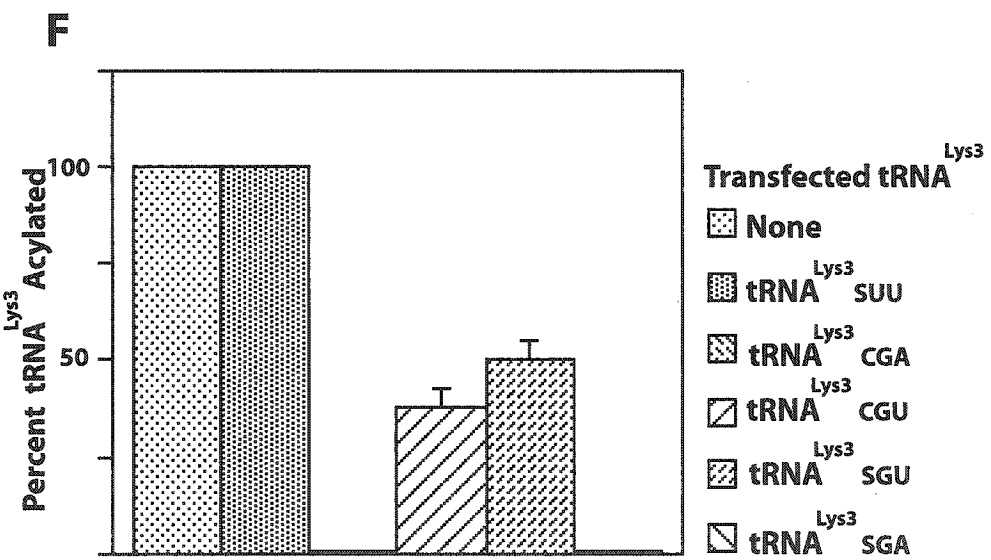
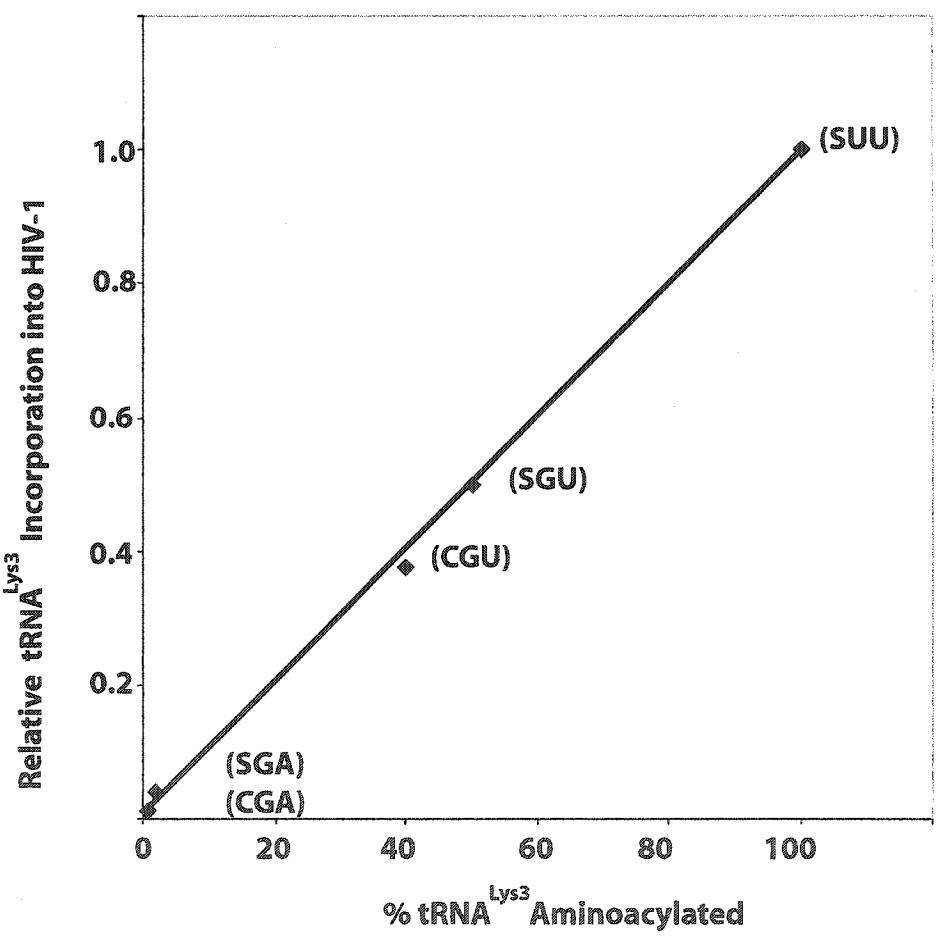

F
Percent tRNA Lys3 Acylated
100
50
Transfected tRNA Lys3
None
tRNA Lys3 SUU
tRNA Lys3 CGA
tRNA Lys3 CGU
tRNA Lys3 SGU
tRNA Lys3 SGA

Figure 2.7. **Graph showing the correlation between $tRNA^{Lys3}$ aminoacylation and incorporation into HIV-1.** Letters in parenthesis indicate the anticodon sequence of the $tRNA^{Lys3}$ variants tested. These data are based on the quantitative results shown in Figures 2.4C and 2.6F.


Relative tRNA^{Lys3} Incorporation into HIV-1
1.0
0.8
0.6
0.4
0.2
(SUU)
(SGU)
(CGU)
(SGA)
(CGA)
0
20
40
60
80
100
% tRNA^{Lys3} Aminoacylated

Chapter 3

Ability of Wild-Type and Mutant Lysyl-tRNA Synthetase to Facilitate $tRNA^{Lys}$ Incorporation into HIV-1

This chapter was adapted from an article submitted to Journal of Journal of virology (2003)

3.1 Preface

In the previous chapter, we showed a correlation between the ability of $tRNA^{Lys3}$ to be aminoacylated and its ability to be incorporated into HIV-1. Because the anti-codon mutations in $tRNA^{Lys3}$ inhibited binding to LysRS, reduced $tRNA^{Lys3}$ aminoacylation indicated a reduced interaction between $tRNA^{Lys3}$/LysRS. Therefore, it was not clear whether aminoacylation itself was required for selective packaging of $tRNA^{Lys}$. In this next study, we constructed two truncated LysRS mutants, one of which has lost its aminoacylation ability, while the other has lost its ability to interact with $tRNA^{Lys}$. COS7 cells were co-transfected with plasmids containing HIV-1 proviral DNA and plasmids coding for wild-type or mutant LysRS. We found that viruses produced from cells overexpressing either wild-type or aminoacylation-deficient LysRS plasmid contained approximately 2 times more $tRNA^{Lys3}$ than viruses produced from cells transfected with HIV-1 proviral DNA alone. Viruses produced from cells expressing the LysRS mutant that had lost its ability to bind $tRNA^{Lys}$ did not contain an increased content of $tRNA^{Lys}$. These results indicate that LysRS binding to $tRNA^{Lys}$ is required for the selective packaging of the $tRNA^{Lys}$ into HIV-1, while the ability of the LysRS to aminoacylate the $tRNA^{Lys}$ is not required.

3.2 Abstract

The major human $tRNA^{Lys}$ isoacceptors, $tRNA^{Lys1,2}$ and $tRNA^{Lys3}$, are selectively packaged into HIV-1 during assembly, where $tRNA^{Lys3}$ acts as a primer for reverse transcription. Lysyl-tRNA synthetase (LysRS) is also incorporated into HIV-1, independently of $tRNA^{Lys}$, via its interaction with Gag, and is a strong candidate for being the signal that specifically targets $tRNA^{Lys}$ for viral incorporation. Expression of exogenous wild-type LysRS in cells results in an approximately 2 fold increase in the viral packaging of both LysRS and $tRNA^{Lys}$. Herein, we show that this increase in $tRNA^{Lys}$ incorporation into virions is dependent upon the ability of LysRS to bind to $tRNA^{Lys}$, but not upon its ability to aminoacylate the $tRNA^{Lys}$. COS7 cells were cotransfected with plasmids coding for both HIV-1 and either wild-type or mutant human LysRS, all of which get incorporated into virions with similar efficiency. However, N-terminally truncated LysRS which binds poorly to $tRNA^{Lys}$ does not increase $tRNA^{Lys}$ packaging into viruses, while C-terminally-truncated LysRS, which binds to, but doesn't aminoacylate, $tRNA^{Lys}$, still facilitates an increase in $tRNA^{Lys}$ packaging into virions.

3.3 Introduction

In HIV-1, $tRNA^{Lys3}$ serves as the primer tRNA for the reverse transcriptase-catalyzed synthesis of minus-strand strong-stop cDNA (137). During HIV-1 assembly, the major $tRNA^{Lys}$ isoacceptors, $tRNA^{Lys1,2}$ and $tRNA^{Lys3}$, are selectively packaged into the virion (122). The viral precursor protein Gag-Pol interacts with $tRNA^{Lys}$, and is known to be involved in $tRNA^{Lys}$ incorporation into viruses or Gag viral-like particles (126, 151, 153). However, the identity of viral or host cell factors that specifically target the $tRNA^{Lys}$ isoacceptors for interaction with viral proteins is less clear. Since the $tRNA^{Lys}$-binding protein, human lysyl-tRNA synthetase (LysRS), is also selectively packaged into the virion (32), it is a prime candidate for facilitating viral packaging of $tRNA^{Lys}$. A viral population contains on average, approximately 20-25 molecules of LysRS/virion (30), similar to the average number of $tRNA^{Lys}$ molecules/virion (110). The incorporation of LysRS into HIV-1 also appears to be specific. Published work (30, 32) indicates that HIV-1 does not contain isoleucyl tRNA synthetase (IleRS), prolyl tRNA synthetase (ProRS), nor trptophanyl tRNA synthetase (TrpRS), while unpublished work (100) further indicates the additional absence in HIV-1 of aminoacy-tRNA synthetases for arginine (ArgRS), glutamine (GlnRS), methionine (MetRS), and tyrosine (TyrRS).

LysRS has been shown to interact directly with Gag in vitro (121), and is packaged efficiently into viral-like particles (VLPs) composed only of Gag (32),

which do not selectively package $tRNA^{Lys}$ due to the absence of Gag-Pol (151). This indicates that LysRS incorporation into Gag particles occurs independently of $tRNA^{Lys}$ packaging, and this conclusion is further supported by the finding that mutant LysRS not containing $tRNA^{Lys}$-binding domains is still incorporated into virions (121). Since Gag and Gag-Pol interact during viral assembly (172, 192), a likely model for $tRNA^{Lys}$ packaging into virions would involve a Gag/Gag-Pol complex interacting with a $tRNA^{Lys}$/LysRS complex, with Gag interacting with Gag-Pol and LysRS, and Gag-Pol interacting with $tRNA^{Lys}$.

Further support for a role of LysRS in $tRNA^{Lys}$ packaging into viruses comes from experiments in which COS7 cells were cotransfected with HIV-1 proviral DNA and a plasmid coding for wild-type LysRS. The expression of exogenous wild-type LysRS in the cell results in a maximum 2-fold increase in the incorporation of both total LysRS (endogenous and exogenous) and $tRNA^{Lys}$ into virions (83). In this work, we utilize this observation to study the effect of the expression of mutant LysRS species upon both LysRS and $tRNA^{Lys}$ incorporation into viruses. We have previously shown that the ability of $tRNA^{Lys3}$ anticodon mutants to be incorporated into HIV-1 was directly correlated with their ability to be aminoacylated (119). However, in that report, aminoacylation was used to measure the ability of the mutant $tRNA^{Lys}$ to bind to LysRS, and it was unclear if aminoacylation of the $tRNA^{Lys}$ was in itself required for $tRNA^{Lys}$ packaging. In this report, we will show that binding of LysRS to $tRNA^{Lys}$ is required for the LysRS-

facilitated increase in $tRNA^{Lys}$ incorporation into virions, but that the ability of LysRS to aminoacylate $tRNA^{Lys}$ is not required.

3.4 Experimental Procedures

3.4.1 Plasmid Construction— SVC21.BH10 P- is a simian virus 40-based vector that contains full-length wild-type HIV-1 proviral DNA containing an inactive viral protease (D25G), and was a gift from E. Cohen, University of Montreal. Protease-negative viruses have been shown to package $tRNA^{Lys}$ into HIV-1 as efficiently as protease-positive viruses (126, 151), and are used here because they generally give clearer and more consistent results than viruses with an active protease, probably because of the large amount of inappropriate proteolysis of Gag and Gag-Pol observed in the cytoplasm of protease-positive HIV-1-infected cells.

Plasmid pM368 contains cDNA encoding full-length (1-597 amino acids) human LysRS, as previously described (190). The cDNA was PCR-amplified, and digested with EcoR1 and Xho1, whose sites were placed in each of the PCR primers. To produce an N-terminal truncated LysRS encoding amino acids 66-597, the sense primer was complementary to a sequence downstream of the sequence encoding the N-teminal amino acids. For expression in COS7 cells, the PCR fragments were cloned into pcDNA 3.1 (Invitrogen) to obtain LysRS.F and LysRS.T, expressing full-length or N-terminally truncated LysRS, respectively. To produce myc-tagged LysRS (Myc-LysRS) we PCR-amplified the full-length LysRS cDNA, and then cloned the fragment into pcDNA 1 (Invitrogen) into which sequences containing myc and His_6 had been inserted (36). This results in a LysRS variant containing both an N-terminal Myc tag and a C-terminal His_6 tag.

To purify the wild-type (LysRS.F) and mutant (LysRS.T) synthetases, the corresponding PCR fragments were cloned into the bacterial expression vector pET-21b (+) (Clonetech), which expresses the proteins with a C-terminal His_6 tag. Both proteins were overexpressed in *Escherichia coli,* and purified as previously described (190).

3.4.2 Cell Culture and Virus Production— COS7 cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum and antibiotic. Transfection of COS7 cells with the above plasmids by the calcium phosphate method was as previously described (151). Viruses were isolated from COS7 cell culture medium 63 h post-transfection, or from the cell culture medium of infected cell lines. The virus-containing medium was first centrifuged in a Beckman GS-6R rotor at 3,000 rpm for 30 minutes and the supernatant was then filtered through a 0.2 μm filter. The viruses in the filtrate were then pelleted by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 1 h. The viral pellet was then purified by centrifugation with a Beckman SW41 rotor at 26,500 rpm for 1 h through 15% sucrose onto a 65% sucrose cushion.

3.4.3 RNA Isolation and Analysis— Total cellular or viral RNA was extracted from cell or viral pellets by the guanidinium isothiocyanate procedure (37), and dissolved in 5 mM Tris buffer, pH 7.5. Hybridization to dot-blots of cellular or viral RNA was carried out with DNA probes complementary to $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ (122), genomic RNA (29), and -actin mRNA (DNA probe from Ambion). Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) of ^{32}pCp-3′-end-labeled viral RNA was carried out as previously described (122).

3.4.4 Western Blotting— Sucrose gradient-purified virions were resuspended in 1X radioimmunoprecipitation assay buffer (RIPA buffer: 10 mM Tris, pH 7.4,100 mM NaCl, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, protease inhibitor cocktail tablets (Boehringer Mannheim)). Western blot analysis was performed using either 300 µg of cellular protein or 10 µg of viral protein, as determined by the Bradford assay (20). The cellular and viral lysates were resolved by SDS-PAGE, followed by blotting onto nitrocellulose membranes (Gelman Sciences). Detection of protein on the Western blot utilized monoclonal antibodies or antisera specifically reactive with viral capsid (CAp24) (mouse antibody, Intracel), -actin (Sigma Aldrich), and human LysRS (rabbit antibody (190)). Western blots were analyzed by enhanced chemiluminescence (ECL kit, Amersham Life Sciences) using goat anti-mouse or donkey anti-rabbit (Amersham Life Sciences) as a secondary antibody. The sizes of the detected protein bands were estimated using pre-stained high molecular mass protein markers (GIBCO/BRL).

3.4.5 Electrophoretic Band Shift Assay— $tRNA^{Lys}$ was purified from human placenta as previously described (122), and labeled with the ^{32}pCp-3′-end-labeling technique as previously described (22). In 20 µl binding buffer (20 mM Tris-HCl, pH 7.4, 75 mM KCl, 10 mM $MgCl_2$, and 5% glycerol) 5 nM labeled $tRNA^{Lys}$ was incubated with different concentrations of either wild-type or truncated human LysRS (0.06 µM, 0.3 µM, or 1.5 µM) for 15 min on ice, and then analyzed by native 6% polyacrylamide gel electrophoresis (PAGE) carried out at 4 °C.

3.4.6. In vitro aminoacylation of tRNALys3 by LysRS. The reactions were conducted at 30^0C, in a final volume of 100 l containing 10 mM of enzyme (wild-type LysRS, 1-65 LysRS, or 452-597 LysRS), 50mM HEPES, pH 7.5, 0.1 mgm/ml bovine serum albumin, 20 mM KCl, 10 mM $MgCl_2$, 20 mM -mercaptoethanol, 4 mM ATP, 20 uM lysine, 0.3 C/ l [^{3}H] lysine (Amersham), and 0.4 mgm/ml calf liver tRNA (Sigma). At two minute intervals, 10 l aliquots were spotted onto trichloroacetic acid (TCA)-soaked Whatman 3MM filters. The filters were washed three times with 10% TCA, and counted by scintillation counting.

3.5 Results

3.5.1 Incorporation of wild-type and mutant LysRS into HIV-1—COS7 cells were cotransfected with a plasmid containing HIV-1 proviral DNA and a plasmid coding for either full-length human LysRS (termed LysRS.F, to distinguish it from endogenous LysRS in COS7 cells) or a truncated LysRS variant in which the N-terminal 65 amino acids have been deleted (Δ1-65 LysRS). Figure 3.1 shows Western blots of cell lysates probed with either anti-LysRS (A) or anti-β-actin (B). The bands were quantitated, and the LysRS/β-actin ratios are shown in panel C, normalized to the LysRS/β-actin ratio in COS7 cells expressing only endogenous LysRS (panels A-C, lane 1). Δ1-65 LysRS is expressed somewhat better than LysRS.F, as shown by the higher LysRS/β-actin ratios (Figure 1C). Lane K contains purified human LysRS tagged at the N-terminal with an MRGSHHHHHHSSGWVD sequence, which contain His_6 used for purifying the LysRS.

The packaging of LysRS into HIV-1 was examined next. Figure 3.1 shows Western blots of viral lysates probed with either anti-LysRS (panel D) or anti-capsid (CA) (panel E). Anti-CA detects the capsid sequences within the unprocessed Gag protein present in protease-negative viruses. The bands were quantitated, and the LysRS/Gag ratios are shown in panel F, normalized to the LysRS/Gag ratio for cells transfected with BH10P- only (panels D-F, lane 1). As previously reported (32), virions incorporating either endogenous LysRS (panel

D, lane 1) or exogenous LysRS.F (panel D, lane 2) contains both full-length and smaller LysRS species. In addition to these species, the Δ1-65 LysRS species can also be seen in the viruses produced from cells expressing this LysRS variant (panel D, lane 3). The expression of exogenous LysRS.F or Δ1-65 LysRS results in increases in the total LysRS incorporated into virions (Figure 3.1F), with more total LysRS incorporated into virions upon expression of Δ1-65 LysRS (Figure 3.1D, lane 3) than upon expression of LysRS.F. This difference may reflect the higher amount of Δ1-65 LysRS present in the cytoplasm (Figure 3.1C).

Factors regulating the ability of exogenous LysRS to be packaged into the virus were further examined through expression of two other mutant LysRS species: A full-length LysRS variant, containing extra amino acids of Myc sequence at the N terminus (Myc-LysRS), and a LysRS mutant missing the C-terminal 145 amino acids (Δ452-597 LysRS), which also contains the N-terminal Myc. The deleted sequences in Δ452-597 LysRS include motif 3, a region believed to participate in the aminoacylation site of the enzyme (27, 51). The cytoplasmic expression of Myc-LysRS and Δ452-597 LysRS in COS7 cells cotransfected with BH10P- is shown in Figure 3.2 by Western blots of cell lysates probed with either anti-LysRS (A) anti-Myc (B), or anti-β-actin (C). In panel A, it can be seen that Myc-LysRS (lane 2) and Δ452-597 LysRS (lane 3) are distinguished from endogenous LysRS (lane 1) by their reduced and increased electrophoretic mobilities, respectively. Panel B shows Western blots probed with anti-Myc, which detects only the expression of exogenous LysRS variants.

The LysRS (panel A): β-actin ratios are plotted in panel D, normalized to the endogenous LysRS:β-actin ratio. Panel A indicates that the majority of cytoplasmic LysRS is either Myc-LysRS or Δ452-597 LysRS. The cytoplasmic expression of Myc-LysRS and Δ452-597 LysRS (Figure 3.2D) appears somewhat greater than that found for Δ1-65 LysRS or LysRS.F (Figure 3.1C). This may be due to the different vectors used to express LysRS.F and Δ1-65 LysRS (pcDNA 3.1) or Myc-LysRS and Δ452-597 LysRS (pcDNA 1.0).

Figure 3.2 also shows Western blots of viral lysates probed with either anti-LysRS (panel E), anti-Myc (panel F), or anti-capsid (panel G). The bands were quantitated, and the total viral LysRS/Gag ratios are shown graphically in panel H, normalized to the LysRS/Gag ratio for cells transfected with BH10P- only (lane 1). The results indicate that both exogenous Myc-LysRS and Δ452-597 LysRS are packaged into virions (panels E,F). We also note that unlike endogenous LysRS in viruses, which is processed to species with increased electrophoretic mobility (Figure 3.1D, lanes 1-3 and Figure 3.2E, lane 1), Myc-LysRS processing is not apparent, as indicated by the strong signal for full-length Myc-LysRS without any significant increase in the smaller band containing processed endogenous LysRS (Figure 3.2E, lane 2).

3.5.2 Effect of the Expression of Exogenous Wild-Type and Mutant LysRS Upon Cytoplasmic and Viral Concentrations of tRNALys Isoacceptors— Figure 3.3 shows the effect of expression of wild-

type and mutant LysRS upon $tRNA^{Lys}$ concentrations in the cytoplasm of HIV-1-transfected COS7 cells and in the virions produced from these cells. Dot-blot hybridization was used to determine the $tRNA^{Lys}$/β-actin mRNA ratios in cellular RNA (panel A), or the $tRNA^{Lys3}$/genomic RNA ratios in total viral RNA (panel B) (122). As shown in Figure 3.3A, we observe small increases (20-25%) in the cytoplasmic concentrations of the major $tRNA^{Lys}$ isoacceptors upon expression of LysRS.F, Δ1-65 LysRS, and Myc-LysRS, while expression of Δ452-597 LysRS induces no such increase. On the other hand, panel B indicates that incorporation of $tRNA^{Lys}$ isoacceptors is increased upon expression of LysRS.F, Myc-LysRS, and Δ452-597 LysRS, but is actually decreased somewhat upon expression of Δ1-65 LysRS.

While expression of wild-type LysRS results in an increase in cytoplasmic $tRNA^{Lys}$ as well, this is not responsible for the increased $tRNA^{Lys}$ packaging into the virion. Thus, an increase in cytoplasmic $tRNA^{Lys}$ resulting from expression of Δ1-65 LysRS does not result in an increase in $tRNA^{Lys}$ incorporation into virions, while the increased packaging of $tRNA^{Lys}$ into virions due to expression of Δ452-597 LysRS is not accompanied by any increase in $tRNA^{Lys}$ in the cytoplasm. The inability of Δ452-597 LysRS to cause an increase in $tRNA^{Lys}$ production may reflect a transcription control mechanism in which the synthesis of new $tRNA^{Lys}$ is regulated by the ratio of aminoacylated:unacylated $tRNA^{Lys}$. In yeast, uncharged $tRNA^{Lys}$ has been shown to act via a signal transduction pathway to activate the synthesis of LysRS through increased transcription of the LysRS gene (134).

Presumably, this transcriptional regulation mechanism will maintain the optimum LysRS/$tRNA^{Lys}$ ratio to keep all $tRNA^{Lys}$ in a charged state, a state that could also be achieved by decreasing synthesis of new $tRNA^{Lys}$.

Overexpression of either $tRNA^{Lys3}$ or $tRNA^{Lys2}$ in the cells containing a plasmid coding for either $tRNA^{Lys}$ isoacceptor will result in an increase in that specific $tRNA^{Lys}$ isoacceptor in the virion. However, the total $tRNA^{Lys}$/virion remains the same, i.e., an increase in one of the $tRNA^{Lys}$ isoacceptors results in a decrease in the other (83). The molecule that initially limits the $tRNA^{Lys}$ /virion appears to be LysRS, since as previously shown (83), and shown herein, expression of LysRS results in an increase in all major $tRNA^{Lys}$ isoacceptors. Presumably, cellular LysRS and $tRNA^{Lys}$ are present in great excess over the amount packaged into virions, since infection of cells with HIV-1 does not noticeably reduce cell replication. It seems likely, therefore, that if LysRS represents an initially limiting factor for $tRNA^{Lys}$ incorporation into HIV-1, the $tRNA^{Lys}$/LysRS complex that interacts with viral proteins may be a small pool separate from the bulk cytoplasmic pool. In fact, recent work indicates that the cellular source of viral LysRS may be newly-synthesized LysRS (96).

The increase in viral $tRNA^{Lys}$ accompanying the increase in viral LysRS incorporation is not accompanied by an increase in Gag-Pol incorporation (83), and the limited increases in both viral LysRS and $tRNA^{Lys}$ we observe may result from limiting amounts of Gag-Pol incorporation. That viral LysRS and $tRNA^{Lys}$

can increase at all implies that there are a restricted number of unsaturated binding sites on the Gag/Gag-Pol complex for these molecules. This would also explain why Δ1-65 LysRS only creates a small decrease in $tRNA^{Lys}$ incorporation, since most of its binding might be to these unsaturated sites, and non-competitive with wild-type endogenous LysRS/ $tRNA^{Lys}$

3.5.3 Ability of LysRS Variants to Bind or Aminoacylate tRNALys3 In Vitro— The ability of some of LysRS variants to bind or aminoacylate $tRNA^{Lys3}$ in vitro was determined to see if this could explain the varying ability of LysRS species to facilitate $tRNA^{Lys}$ packaging. LysRS.F, 1-65 LysRS, and 452-597 LysRS, tagged at their C-terminus with His_6, were partially purified from transformed bacteria using Ni^+ chromatography. Their relative binding affinities to human placental $tRNA^{Lys3}$ were compared using an electrophoretic band shift assay. Human $tRNA^{Lys3}$ was 3′-end labeled with ^{32}pCp (22), and incubated with increasing amounts of each LysRS species. The resulting complexes were resolved on a native polyacrylamide gel. As shown in Figure 3.4A, 1-65 LysRS shows significantly reduced binding to $tRNA^{Lys3}$ compared to LysRS.F, while binding of 452-597 LysRS is slightly less compared to that of LysRS.F. Thus the relative binding affinities of the different LysRS variants are reflected in their ability to facilitate incorporation of $tRNA^{Lys}$ into virions. The reduced affinity of 1-65 LysRS for human placental $tRNA^{Lys3}$ in vitro is consistent with a report that an N-terminally truncated form of hamster

LysRS displayed significantly reduced (about 100-fold) affinity for in vitro-synthesized $tRNA^{Lys3}$ transcripts relative to the full-length enzyme (76).

3.6 Discussion

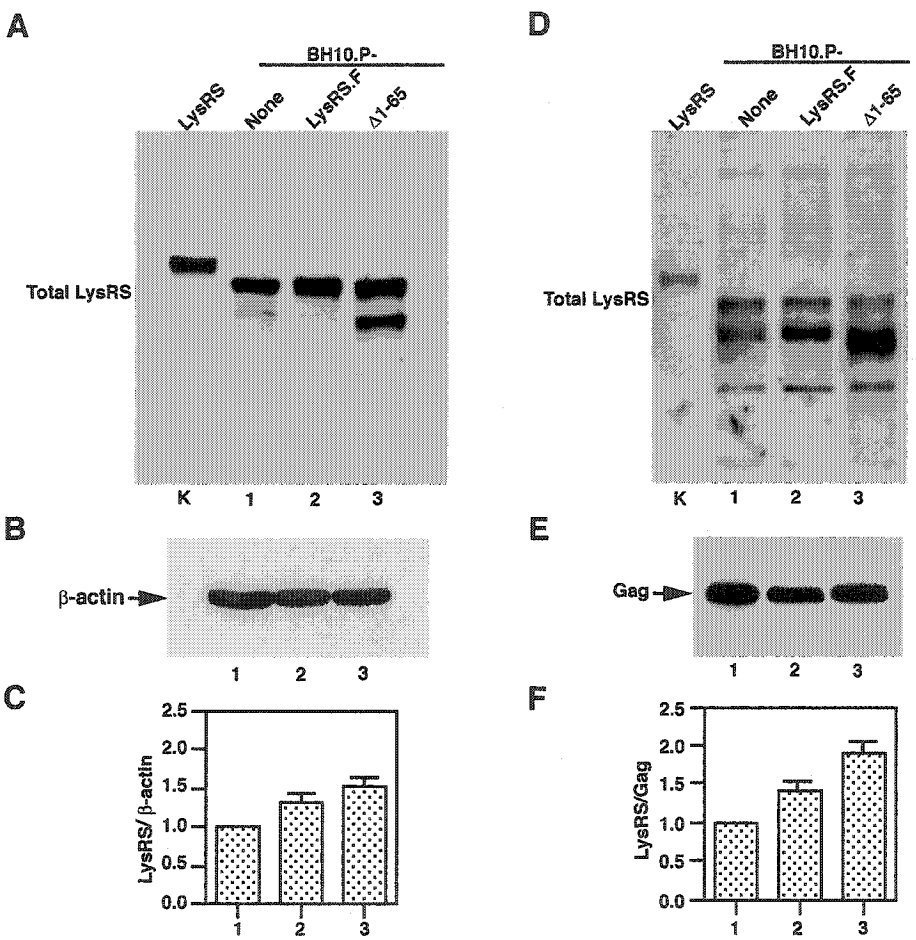
A correlation between the ability of $tRNA^{Lys}$ to be packaged into viruses, and its ability to be aminoacylated in vivo has been reported (119). In that work, COS7 cells were cotransfected with HIV-1 proviral DNA and plasmids coding for anticodon mutants of $tRNA^{Lys3}$. The ability of these mutant tRNAs to be aminoacylated in vivo was correlated with their ability to be packaged into HIV-1. Since the $tRNA^{Lys}$ anticodon is an important site for interaction with LysRS (52), aminoacylation was used as an indication of $tRNA^{Lys3}$ binding to endogenous LysRS, and the correlation showed the importance of LysRS binding to $tRNA^{Lys3}$ for its incorporation into virus. But whether aminoacylation itself is required for $tRNA^{Lys3}$ packaging could not be determined from those experiments.

The Δ452-597 LysRS lacks motif 3, whose sequences contribute to the catalytic aminoacylation site (27, 51). We therefore tested the ability of this and other mutant LysRS species to aminoacylate $tRNA^{Lys3}$ *in vitro* by using these enzymes to aminoacylate calf liver tRNA (Sigma) with ^{3}H-lysine in a standard aminoacylation reaction. The aminoacylation time course for three LysRS variants is shown in Figure 3.4B. As expected, Δ452-597 LysRS is severely inhibited in its aminoacylation ability. Thus the ability of LysRS to facilitate $tRNA^{Lys}$ packaging into virions is not correlated with the enzyme's ability to aminoacylate $tRNA^{Lys3}$. Δ1-65 LysRS, although having a weaker binding affinity

for $tRNA^{Lys3}$, still shows an intermediate ability to aminoacylate $tRNA^{Lys3}$. This is supported by a report that indicated that the N-terminal domain of hamster LysRS, while not essential for aminoacylation, improves the docking of the acceptor arm of $tRNA^{Lys3}$ into the active site of the enzyme (76).

In HIV-1, the $tRNA^{Lys3}$ is not acylated (111), which is probably required to allow the terminal 3` adenosine of $tRNA^{Lys3}$ to be extended by reverse transcriptase. It is not known if only uncharged cytoplasmic $tRNA^{Lys}$ is targeted by viral protein for incorporation into viruses, or if charged $tRNA^{Lys}$ is deacylated after binding to viral proteins. Thus, it is possible that a Gag/LysRS complex might bind to uncharged $tRNA^{Lys}$ without acylating it, or, instead, Gag might induce deacylation of the charged $tRNA^{Lys}$ within a $tRNA^{Lys}$ /LysRS complex.

Figure 3.1 **Effect of expression of wild-type or N-terminal truncated LysRS upon the cytoplasmic and viral concentrations of LysRS**. (A-C) Cytoplasmic concentration of LysRS. Western blot analysis of COS7 cell lysates, probed with either anti-LysRS (A) or anti-β-actin (B). Panel C shows the LysRS/β-actin ratio, determined by quantitative analysis of the bands in panels A and B. Lane K, purified His_6-tagged human LysRS, containing the appended N-terminal MRGSHHHHHHSSGWVD sequence (32). The other lanes represent COS7 cells transfected with the following plasmids: 1, BH10P-; 2, BH10P- and LysRS.F; 3, BH10P- and Δ1-65 LysRS. (D-F) Viral concentrations of LysRS. Western blot analysis of viral lysates probed with anti-LysRS (D) or anti-CA (E). Panel F shows the LysRS/Gag ratio determined from the data in panels D and E. Lanes 1-3 represent viruses produced from COS7 cells transfected with the following plasmids: 1, BH10P-; 2, BH10P- and LysRS.F; 3, BH10P- and Δ1-65 LysRS. Lane K, purified His_6-tagged human LysRS. The bar graphs in panels C and F represent the means of experiments performed three times or more, and the error bars represent standard deviations.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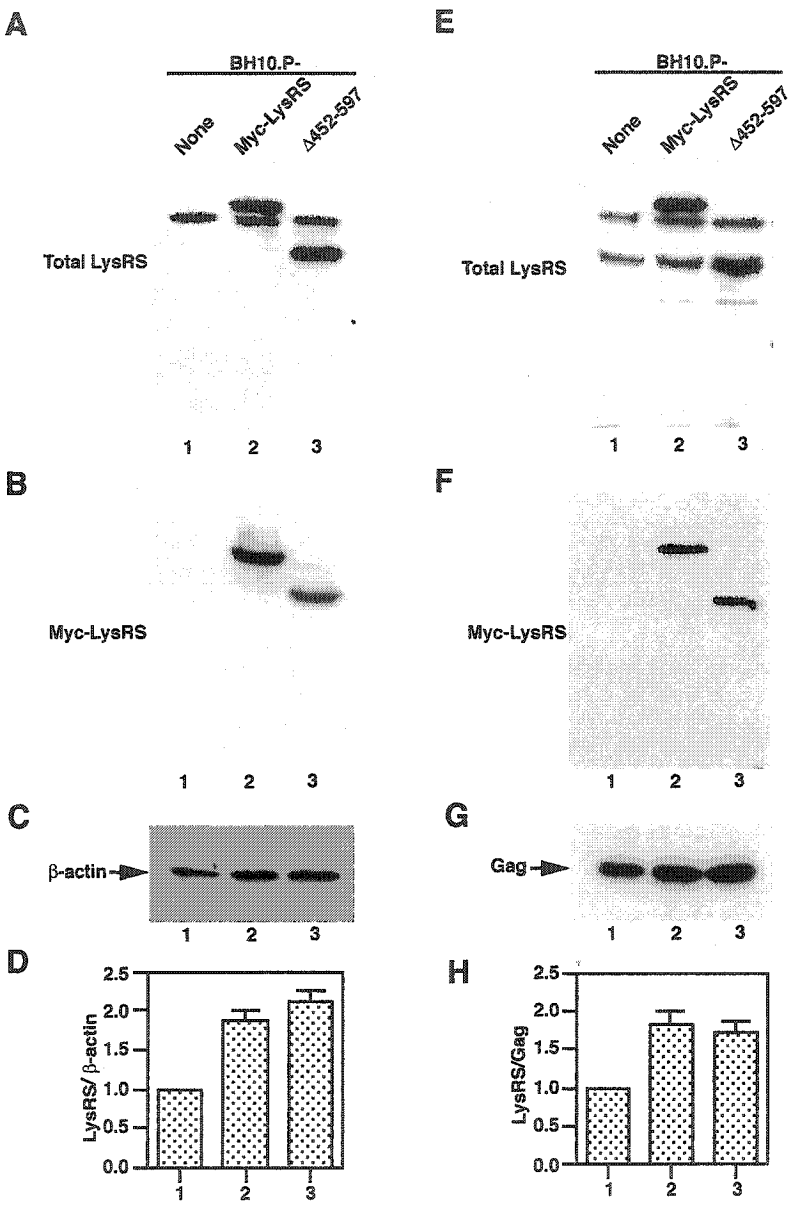
BH10.P-
LysRS
None
LysRS.F
Δ1-65
Total LysRS
K
1
2
3
D
BH10.P-
LysRS
None
LysRS.F
Δ1-65
Total LysRS
K
1
2
3
B
β-actin
1
2
3
E
Gag
1
2
3
C
LysRS/ β-actin
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
F
LysRS/Gag
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3

Figure 3.2. **Effect of expression of Myc-LysRS and Δ452-597 LysRS upon the cytoplasmic and viral concentrations of LysRS.** (A-D) Cytoplasmic concentrations of LysRS. Western blot analysis of COS7 cell lysates, probed with either anti-LysRS (A), anti-Myc (B), or anti-β-actin (C). Panel D shows the LysRS/β-actin ratio, determined by quantitative analysis of the bands in panels A and C. The lanes in each panel represent COS7 cells transfected with the following plasmids: 1, BH10P-; 2, BH10P- and Myc-LysRS; 3, BH10P- and Δ452-597 LysRS. Myc-LysRS contains the Myc-containing sequence (MASMEQKLISEEDLNNG) appended to the N terminus of LysRS. (E-H) Western blots probing viral concentrations of LysRS. Viral lysates were probed with either anti-LysRS (E), anti-Myc (F), or anti-CA (G). Panel H shows the LysRS/Gag ratio determined from the data in panels E and G. Lanes in panels E-H represent viruses produced from cells transfected with: 1, BH10P-; 2, BH10P- and Myc-LysRS; 3, BH10P- and Δ452-597 LysRS. The bar graphs in panels D and H represent the means of experiments performed three times or more, and the error bars represent standard deviations.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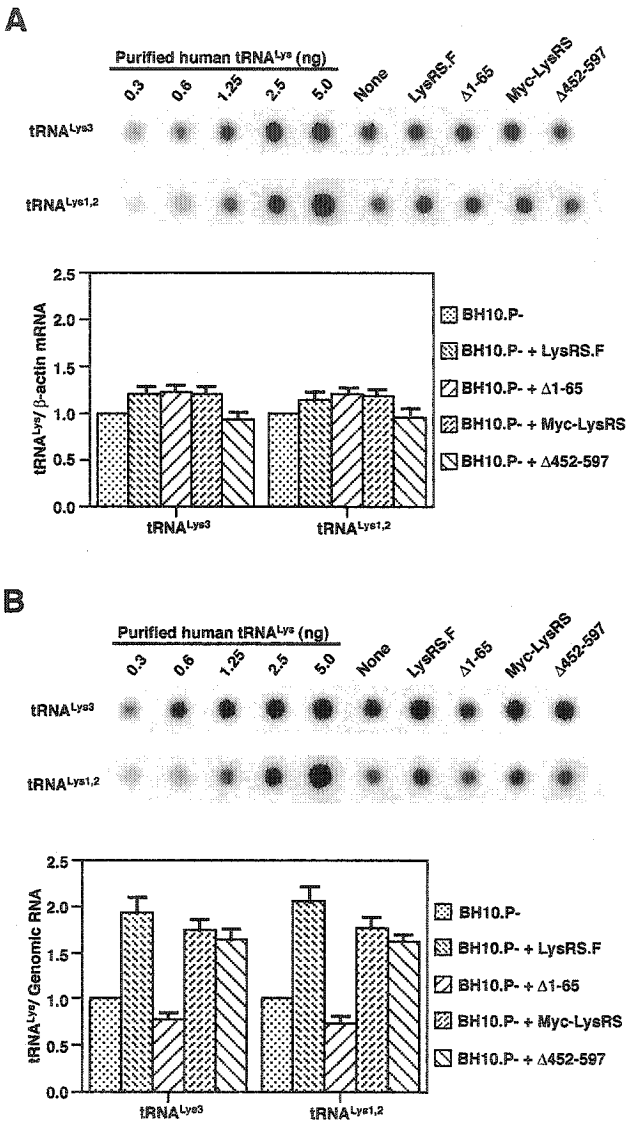
BH10.P-
None
Myc-LysRS
Δ452-597
Total LysRS
1
2
3
E
BH10.P-
None
Myc-LysRS
Δ452-597
Total LysRS
1
2
3
B
Myc-LysRS
1
2
3
F
Myc-LysRS
1
2
3
C
β-actin
1
2
3
G
Gag
1
2
3
D
LysRS/β-actin
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
H
LysRS/Gag
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3

Figure 3.3. **Effect of expression of wild-type or mutant LysRS upon the cellular and viral concentrations of $tRNA^{Lys}$.** Dot-blots of total cellular (A) or total viral (B) RNA were hybridized with DNA probes to either β-actin mRNA (A) or viral genomic RNA (B), and to either $tRNA^{Lys3}$ or $tRNA^{Lys1,2}$ (A, B). In the dot blots shown, the samples contained equal amounts of β-actin mRNA (A) or viral genomic RNA (B). Dot blots were analyzed by phosphorimaging, and the ratios of $tRNA^{Lys}$/β-actin mRNA (A) and $tRNA^{Lys}$/genomic RNA (B) were determined for cells cotransfected with BH10P- and one of the following LysRS-encoding plasmids: none, LysRS.F, Δ1-65 LysRS, Myc-LysRS, or Δ452-597 LysRS. The bar graphs in panels A and B represent the means of experiments performed three times or more, and the error bars represent standard deviations.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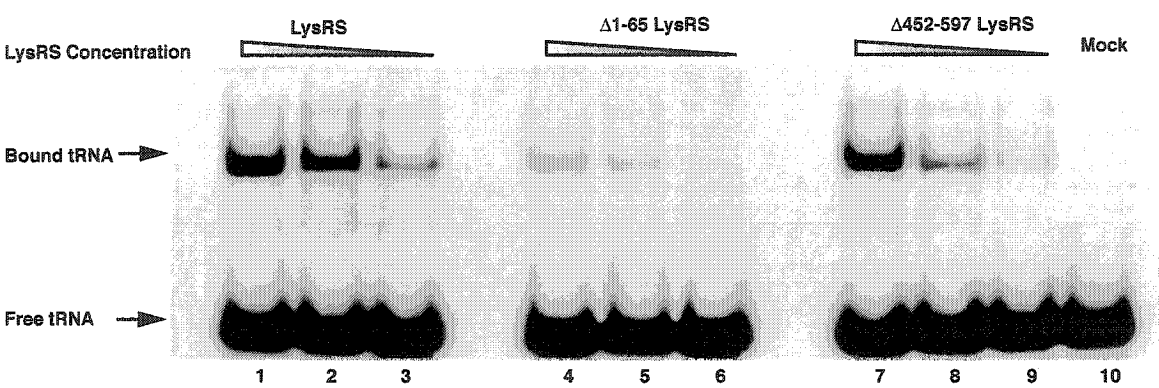
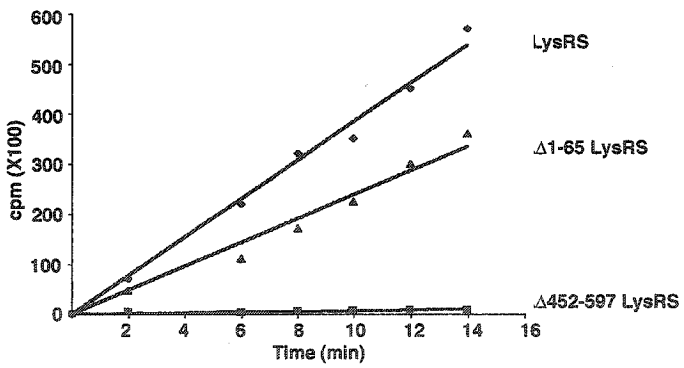
0.3
0.6
1.25
2.5
5.0
None
LysRS.F
Δ1-65
Myc-LysRS
Δ452-597
tRNALys3
tRNALys1,2
tRNALys/ β-actin mRNA
2.5
2.0
1.5
1.0
0.5
0.0
BH10.P-
BH10.P- + LysRS.F
BH10.P- + Δ1-65
BH10.P- + Myc-LysRS
BH10.P- + Δ452-597
tRNALys3
tRNALys1,2
B
Purified human tRNALys (ng)
0.3
0.6
1.25
2.5
5.0
None
LysRS.F
Δ1-65
Myc-LysRS
Δ452-597
tRNALys3
tRNALys1,2
tRNALys/ Genomic RNA
2.5
2.0
1.5
1.0
0.5
0.0
BH10.P-
BH10.P- + LysRS.F
BH10.P- + Δ1-65
BH10.P- + Myc-LysRS
BH10.P- + Δ452-597
tRNALys3
tRNALys1,2

Figure 3.4. **Interaction and aminoacylation of $tRNA^{Lys3}$ with wild-type and mutant LysRS *in vitro*.** A. The interaction between $tRNA^{Lys3}$ and wild-type LysRS, Δ1-65 LysRS, or Δ452-597 LysRS was measured using an electrophoretic band shift assay. B. *In vitro* aminoacylation of $tRNA^{Lys3}$ by LysRS.

A


LysRS
Δ1-65 LysRS
Δ452-597 LysRS
LysRS Concentration
Mock
Bound tRNA
Free tRNA
1 2 3 4 5 6 7 8 9 10

B


600
500
400
300
200
100
0
cpm (X100)
2 4 6 8 10 12 14 16
Time (min)
LysRS
Δ1-65 LysRS
Δ452-597 LysRS

Chapter 4

The Interaction Between HIV-1 Gag and Human Lysyl-tRNA Synthetase During Viral Assembly

This chapter was adapted from an article published in Journal of biological chemistry (2003. In press)

4.1 Preface

Lysyl-tRNA-synthetase is specifically incorporated into HIV-1, and among viral proteins, Gag is sufficient for this incorporation. In this study, we have mapped the sites of interaction between HIV-1 Gag and LysRS. Using deletion mutants, we studied the interaction between purified Gag and LysRS *in vitro* using GST-pulldown assays, and *in vivo* by measuring the incorporation of LysRS into Gag VLPs. We found that Gag sequences within the C-terminal domain of CA and LysRS sequences which include motif 1 are important for the Gag/LysRS interaction.

4.2 Abstract

Human lysyl-tRNA synthetase (LysRS) is a tRNA binding protein that is selectively packaged into HIV-1 along with its cognate $tRNA^{Lys}$ isoacceptors. Evidence exists that Gag alone is sufficient for the incorporation of LysRS into virions. Herein, using both *in vitro* and *in vivo* methods, we begin to map regions in Gag and LysRS that are required for this interaction. *In vitro* reactions between wild-type and truncated HIV-1 Gag and human LysRS were monitored using GST-tagged molecules and glutathione agarose chromatography. Gag /LysRS interaction *in vivo* was detected in 293FT cells cotransfected with plasmids coding for wild-type or mutant HIV-1 Gag and LysRS, either by monitoring Gag /LysRS complexes immunoprecipitated from cell lysate with anti-LysRS, or by measuring the ability of LysRS to be packaged into budded Gag viral-like particles. Based on these studies, we conclude that the Gag/LysRS interaction depends upon Gag sequences within the C-terminal domain of capsid (the last 54 amino acids) and amino acids 208-259 of LysRS. The latter domain includes the class II aminoacyl-tRNA synthetase consensus sequence known as motif 1. Both regions have been implicated in homodimerization of capsid and LysRS, respectively. Sequences falling outside these amino acid stretches can be deleted from either molecule without affecting the Gag /LysRS interaction, further supporting the observation that LysRS is incorporated into Gag viral-like particles independently of its ability to bind $tRNA^{Lys}$.

4.3 Introduction

The life cycle of HIV-1 has been intensely studied (see (197) for a recent review). Upon infection of a cell by HIV-1, the viral RNA genome is copied into a double-stranded cDNA by the viral enzyme reverse transcriptase (RT). $tRNA^{Lys3}$ is required to initiate reverse transcription (153). The resultant viral DNA is translocated into the nucleus of the infected cell where it integrates into the host cell's DNA, and codes for viral mRNA and proteins. Proteins comprising the viral structure include both the glycosylated envelope proteins (gp120 and gp41), and mature proteins resulting from the processing of the large precursor protein, Gag ($Pr55^{gag}$): matrix (MAp11), capsid (CAp24), and nucleocapsid (NCp7). Gag also contains C-terminal sequences for the p6 protein, which is believed to play a role in viral budding from the cell. The three viral enzymes used in the HIV-1 life cycle result from the processing of another precursor Gag-Pol ($Pr160^{gag\text{-}pol}$), and are: protease (PRp11), reverse transcriptase (RTp66/p51), and integrase (INp32). Both Gag and Gag-Pol are translated from the same full-length viral RNA, and this RNA, which also serves as the viral genomic RNA, is packaged into assembling virions via binding to nucleocapsid sequences in Gag(14, 92). The in vivo interaction of Gag with Gag-Pol has also been well-documented (172, 191-193), and Gag-Pol is carried into the assembling Gag particle by its interaction with Gag, probably through intermolecular interactions between homologous Gag sequences. The Gag and Gag-Pol proteins assemble at the cell membrane, and during budding from the cell, the viral protease, PRp11, is activated and cleaves these two precursor precursors into the proteins found in the mature virion.

The major $tRNA^{Lys}$ isoacceptors in mammalian cells, $tRNA^{Lys1,2}$ and $tRNA^{Lys3}$, are also selectively packaged into the virion during its assembly (122). Gag protein is capable of forming extracellular Gag viral-like particles (VLPs), which are made by transfecting cells with a plasmid coding only for the Gag, but the additional presence of Gag-Pol is required for the packaging of $tRNA^{Lys}$ into either Gag VLPs or into HIV-1 (151). Increasing the amount of $tRNA^{Lys3}$ incorporated into HIV-1 results in a viral population with increased levels of $tRNA^{Lys3}$ annealed to the viral RNA genome and increased infectivity (83). In addition to the $tRNA^{Lys}$ isoacceptors, human lysyl-tRNA synthetase (LysRS), the enzyme that aminoacylates $tRNA^{Lys}$, is also selectively packaged into HIV-1 during its assembly (30, 32), and is a strong candidate for being the signal by which viral proteins recognize and selectively package the $tRNA^{Lys}$ isoacceptors. The packaging of LysRS into HIV-1 appears to be quite selective. Published work indicates that human IleRS, ProRS, and TrpRS are not detected in the virion (30, 32), while unpublished work in one of our laboratories (L.K.) indicates the additional absence of human ArgRS, GlnRS, MetRS, TyrRS, and AsnRS. In addition, Rous sarcoma virus, which uses $tRNA^{Trp}$ as a primer tRNA for reverse transcription, contains TrpRS, but not LysRS (30). An HIV-1 population contains, on average, approximately 20-25 molecules of LysRS/virion (30), similar to the average number of $tRNA^{Lys}$ molecules/virion (110).

Our current hypothesis for the formation of a $tRNA^{Lys}$ packaging complex includes a Gag /Gag-Pol complex interacting with a $tRNA^{Lys}$/LysRS complex, with Gag interacting with LysRS and Gag-Pol interacting with $tRNA^{Lys}$. In addition to the reports cited above which provide evidence for an interaction between Gag and Gag-Pol, evidence supporting this model includes the following: 1) While the incorporation of $tRNA^{Lys}$ into viruses requires Gag-Pol (151), the incorporation of LysRS into HIV-1 occurs independently of $tRNA^{Lys}$ packaging, i.e., it is also packaged efficiently into Gag VLPs (32), which do not selectively package $tRNA^{Lys}$ (151); 2) Overexpression of LysRS in the cell results in a near doubling of the incorporation of both $tRNA^{Lys}$ and LysRS into HIV-1 (83); 3) The ability of $tRNA^{Lys}$ to interact with LysRS is required for the incorporation of $tRNA^{Lys}$ into the virion (119). Therefore, the interaction between Gag and LysRS may be critical for the selective packaging of primer $tRNA^{Lys3}$ into the virion, and represents a potentially new target for anti-HIV-1 therapy.

The sites of interaction between Gag and LysRS are explored in this report, using both in vitro and in vivo approaches. As described above, the amino acid sequences within the viral precursor that represent different mature viral proteins have been well delineated. Furthermore, the relatively high sequence conservation among LysRSs and the large amount of structural and biochemical data on aminoacyl-tRNA synthetases has greatly facilitated the design of the truncated LysRS constructs used in these studies. The crystal structure of *Escherichia coli* LysRS (169) and *Thermus thermophilus* LysRS (52) have been solved. Eukaryotic LysRS is a class II synthetase, forming a closely related sub-

group (known as IIb) with aspartyl- and asparginyl-tRNA synthetases (67, 68). The anticodon is a major recognition element for all class IIb synthetases, including human LysRS (184, 196). This sub-class is characterized by an N-terminal anticodon binding domain, with a topology known as an oligonucleotide-binding (OB) fold, which is positioned downstream of the N-terminal extension found in higher eukaryotes. While truncation of this extra domain (N-terminal 65 residues) does not significantly affect aminoacylation by human LysRS (190), it has been shown that the N-terminally truncated enzyme does display significantly weaker tRNA binding affinity. Thus, hamster LysRS was determined to have 100-fold lower apparent affinity for $tRNA^{Lys}$ when the N-terminal domain was removed (75), and specific residues within the N-terminus that function in tRNA-binding have recently been identified (77) This domain was proposed to provide hamster LysRS with non-specific tRNA binding properties. All class II synthetases are also characterized by an anti-parallel β-sheet active site fold, and contain three consensus motifs known as motifs 1, 2, and 3 (67). Motif 1 is part of the dimer interface (class II synthetases are dimers or tetramers), whereas motifs 2 and 3 together constitute the aminoacylation active site. The distribution of these different subdomains in LysRS is shown in cartoon form in Figure 4.1A.

4.4 Experimental Procedure

4.4.1 Plasmid construction— pSVGag-RRE and CMV-REV were donated by D. Rekosh and M.L. Hammarskjold (191). Plasmid ZWt, ZWt-p6, and ΔZ-Wt-p6 were constructed as previously described (1). Gag deletion mutants for expression in 293FT cells were constructed by PCR-amplification of the pSVGag-RRE cDNA, and digested with *SalI* and *SpeI*, whose sites were placed in each of the PCR primers. These fragments were cloned into *SpeI-SalI* sites of pSVGag-RRE. The following primers were used to construct these Gag mutants: Δ323-500 (forward primer: 5′-AATCAGTCTAGACAAAATTACCCTATAGTGCAG; reverse primer: 5′-ACTCTGATCACT ATCATTGGACCAACAAGGTTTCTGT. Δ363-500: forward primer: 5′-AATCAGTCTAGA CAAAATTACCCTATAGTGCAG; reverse primer: 5′-ACTCTGATCAATCACAAAACTCTT GCCTTATGGCC. These plasmids express truncated Gag when co-transfected with CMV-REV in 293FT cells.

All the wild type and mutant GST (glutathione S-transferase)-Gag plasmids were constructed using PCR. The pSVGag-RRE cDNA was PCR-amplified and digested with *Eco*RI , whose sites were introduced in each of the PCR primers. These fragments were cloned into the *Eco*RI site of pGEX4t2 (Amersham Pharmacia Biotech). The following primers were used to construct wild-type and mutant GST-Gag: Wild-type: forward primer: 5′-AATTATGAATTCCTATTATTGTGACGAGGGGTCG TTGCC. reverse primer: 5′-

AATTATGAATTCCTATTATTGTGACGAGGGGTCGTTGCC. Δ1-307: 5′-CTCCGGGAATTCCCGCTTCACAGGAGGTAAAAAATT. Δ1-337: 5′-CTC CGG GAATTCCCGGACCAGCGGCTACACTAGAAGA Δ1-378: 5′-CTCCGGGAATTCCCATGC AGAGACGCAATTTTAGGAAC. Δ363-500: 5′-AATTATGAATTCCTACAAAACTCTTGCC TTATGGCC. Δ433-500: 5′-AATTATGAATTCCTACCCTAAAAAATTAGCCTGTCTC. These plasmids express wild-type and truncated Gag in BL21 *Escherichia coli* cells.

Plasmid pM368 contains cDNA encoding full-length (1 to 597 amino acids) human LysRS, as previously described (190). In order to construct wild-type and mutant LysRS, this cDNA was PCR-amplified and digested with *Eco*RI, whose sites were placed in each of the PCR primers. These fragments were cloned into the *Eco*RI site of pcDNA1.0 Myc (Invitrogen). We used the following primers: wild-type LysRS: forward primer: 5′-CTCCGGGAATTCTAGCGGCCGTGCAGGCGGCCGAGGTG. reverse primer: AATTATGAATTCCTAGACAGAAGTGCCAACTGTTGTGCT. Δ452-597: 5′-AATT ATGAATTCCTACAGGAACTCCCCAACAAGCTTGTCAAGGAG. Δ309-597: 5′-AATTA TGAATTCCTAACCAACCACAAGCATCTTATGATAGAGTTC. Δ267-597: 5′-AATTA TGAATTCCTACTATTCAATCTCTAGGAATCCCAG. Δ260-597: 5′-AATTATGAATTCCTA CTAATCTAAGAAACTTCTTATATA. Δ249-597: 5′-AATTATGAATTCTACTACTTAGAG CGGATGATAAATTTCTG. Δ207-597: 5′-AATTATGAATTCCTAAGACAGCAGTGTGAT TCATACGGAATGAT. Δ1-207: 5′-CTCCGGGAATTCTCCCTGTTTGCATATGTTACCTCA TCTTCA. The resulting

constructs express Myc-tagged wild-type and mutant LysRS proteins once transfected into 293FT cells.

All wild-type and mutants GST-LysRS plasmids were constructed using PCR. The cDNA was PCR amplified and digested with *Eco*RI, whose sites were introduced in each of the PCR primers. These fragments were cloned into an *Eco*RI site of pGEX4t2 (Amersham Pharmacia Biotech). The following primers were used to construct wild-type and mutant GST-LysRS: wild-type GST-LysRS: forward primer: 5′-CTCCGGGAATTCT AGCGGCCGTGCAGGCGGCCG AGGTG; reverse primer: 5′-AATTATGAATTCCTAGA CAGAAGTGCCAACTGTTGTGCT. Using the same forward primer, the following reverse primers were used for C-terminal deletions: Δ506-597: 5′-AATTATGAATTCCTACTACATGGGATCATTCAGGTCAGTAT. Δ452-597: 5′-AATTATG AATTCCTACAGGAACTCCCCAACAAGCTTGTCAAGGAG. Δ373-597: 5′-AATTATGAA TT CCTACTAGTAGGTGACCTTGTAACTGCCTGT. Δ309-597: 5′-AATTATGAATTCCTAACC AACCACAAGCATCTTATGATAGAGTTC. Δ249-597: 5′-AATTATGAATTCCTACTACTTAG AGCGGATGATAAATTTCTG. Δ207-597: 5′-AATTATGAATTCCTAAGACAGCAGT GTGAT CTCATACGGAATGAT. The resulting constructs express wild-type and mutant GST-LysRS proteins in BL21 *Escherichia coli* cells.

4.4.2 Production of Wild-type and Mutant HIV-1 Virus— 293FT cells (Invitrogen) are a clonal derivative of the human kidney 293T cell line. They were transfected with wild-type or mutant Gag and LysRS constructs using

Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cell culture supernatant was collected 63 h post-transfection. Gag viral-like particles (VLPs) were pelleted from culture medium by centrifugation in a Beckman 45 Ti rotor at 35,000 rpm for 1 h. The pellet was then purified by centrifugation in a Beckman SW41 rotor at 26,500 rpm for 1 h through 15% sucrose onto a 65% sucrose cushion. The band of purified VLP was removed and pelleted in 1x TNE in a Beckman 45 Ti rotor at 40,000 rpm for 1 h.

4.4.3 Protein analysis— Viral and cellular proteins were extracted with radioimmuno- precipitation assay buffer (10 mM Tris, pH 7.4; 100 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 1% NP-40; 2 mg of aprotinin/ml; 2 mg of leupeptin/ml; 1 mg of pepstatin A/ml; 100 mg of phenylmethylsulfonyl fluoride/ml). The viral and cell lysates were analyzed by SDS-PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amersham Pharmacia Biotech). Detection of protein by Western blotting utilized monoclonal antibodies that are specifically reactive with HIV-1 capsid (Zepto Metrocs, Inc), a polyclonal antibody for human LysRS (Pocono Rabbit Farm and Laboratory, Inc.), a monoclonal antibody for Myc (Invitrogen), a monoclonal antibody for GST (Amersham Pharmacia Biotech), a monoclonal antibody to β-actin (Sigma), and a monoclonal antibody to C-terminal of HIV-1 capsid, which was used to detect ΔZWt-p6 (NIH AIDS Research and Reference Reagent Program). Detection of HIV proteins was performed by enhanced chemiluminescence (NEN Life Sciences Products) using the following secondary antibodies obtained from Amersham Pharmacia Biotech: anti-mouse (for capsid

and Myc), anti-rabbit (for LysRS), anti-goat (for GST), and anti human (for C-terminal capsid).

4.4.4 Bacterial expression and in vitro binding assay— GST-Gag, GST-LysRS and GST control proteins were expressed in *Escherichia coli* BL21 (Invitrogen). The recombinant proteins were induced with 1 mM IPTG at 30°C for 3 hrs. Bacteria were pelleted, washed in STE buffer and resuspended in TK buffer (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 5% glycerol). The suspended bacteria were sonicated for 30 seconds on ice. Insoluble materials were centrifuged at 13000 X *g* for 10 minutes. The supernatant was used for GST-pulled down experiments. Twenty µl of a 50% (vol/vol) slurry of glutathione-agarose beads (Sigma) were prepared as described according to the manufacturer's instructions (Amersham Pharmacia Biotech). The supernatants from wild-type and mutant GST-Gag and GST-LysRS were added to 20 ul of a 50% (vol/vol) slurry of glutathione-agarose beads at 4°C for 1hr. Beads were washed twice with TK buffer plus 500 mM NaCl, and once with TK buffer alone. Beads containing recombinant proteins were resuspended into 150 µl reaction volume with TK buffer. 3 µg of purified Gag (NIH AIDS Research and Reference Reagent Program), or 4 µg of purified His_6-LysRS, were added to each reaction. Reactions were incubated overnight at 4°C. Beads were washed three times with TK buffer and resuspended with 40 µl of 2X loading buffer (50 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 5 minutes and pelleted. Supernatant (30 µl) was

subjected to Western blot analysis for detecting the bound protein and 5 ul of supernatant was used to detect GST fusion protein.

4.4.5 Immunoprecipitation of LysRS/Gag— 293FT cells were removed from the plate and washed with PBS 63 hours post transfection. 293FT cells from 100 mm plates were lysed in 500 μl TNT buffer. Insoluble material was pelleted at 1800 X *g* for 30 minutes. The supernatant was used for immunoprecipitation. Anti-LysRS was first cross-linked to Sepharose beads. 40 μl of antibody and 400 μl of 50% (w/v) protein A-Sepharose (Pharmacia) were incubated together in 10 ml of 0.2 M triethanolamine pH 9. Dimethyl pimelimidate (DMP) cross-linker (Pierce) was then added to a final concentration of 20 mM, and the mixture was incubated for 1 hour at room temperature. The beads were then washed with 5 ml of 0.2 M triethanolamine pH 9, and further incubated in 10 ml of 0.2 M triethanolamine for another 2 hours at room temperature. Equal amounts of protein (approximately 200 - 500 μg, as determined by the BioRad assay) were incubated with 30 μl antibody cross-linked to protein A-Sepharose for 1 hour at 4°C. The immunoprecipitate was then washed three times with TNT buffer and twice with phosphate-buffered saline (PBS). After the final supernatant was removed, 30 μl of 2X sample buffer (120 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, and 0.02% bromphenol blue) was added and the precipitate was then boiled for 5 minutes to release the precipitated proteins. After microcentrifugation, the resulting supernatant was analyzed using Western blots.

4.5 Results

4.5.1 Interaction of mutant and wild-type LysRS with wild type Gag in vitro— Figure 4.1 shows a schematic diagram of the domain architecture of human LysRS. This enzyme contains the three consensus sequences (motifs 1, 2 and 3) common to all class II synthetases, as well as an N-terminal extension proximal to the anticodon binding domain (190). Wild-type LysRS and C-terminally-truncated LysRS variants were tagged with GST on the N-terminus and expressed in *E. coli*. The *E. coli* lysates were adsorbed to glutathione-agarose beads followed by 3 washing steps with buffer containing 20 mM Tris-HCl [pH 7.5], 100 mM NaCl. The washed beads were then incubated in binding buffer containing purified recombinant HIV-1 Gag. The mixture was then washed 3 times in buffer containing 100 mM NaCl and 2 times in buffer containing 200 mM NaCl. Beads were then resuspended directly in SDS sample buffer, boiled, and subjected to SDS-PAGE. Western blots were probed with either anti-GST or anti-Capsid to detect Gag. Figure 4.1A shows the wild-type and mutant LysRS constructs tested, and lists the relative amount of Gag bound to mutant LysRS, where the Gag/LysRS ratio for wild-type LysRS is given a value of 1.00. The Western blot data supporting these results are shown in panels B and C. Panel B shows a Western blot of the gel probed with anti-LysRS. The first lane (GST) represents a control experiment performed with GST alone, while the other lanes show the wild-type and mutant forms of LysRS

eluted from the beads. In panel C, a Western blot of the same sample was probed with anti-Capsid. This data shows that removal of the C-terminal 288 amino acids from LysRS (full length = 597 amino acids) did not prevent Gag binding, but further removal of an additional C-terminal 60 amino acids resulted in severely reduced binding. These data suggest that the sequence between amino acids 249-309 in motif I of LysRS is required for binding to Gag *in vitro*.

4.5.2 Incorporation of mutant and wild-type Myc-tagged LysRS into Gag viral-like particles in vivo— We next tested truncated LysRS variants for their capability to be packaged into Gag viral-like particles (VLPs) *in vivo*. 293FT cells were cotransfected with a plasmid coding for wild-type HIV-1 Gag and a plasmid coding for wild type or N or C-terminal deleted LysRS, tagged with Myc. The expression in the cell of the different LysRS species was determined and the results are shown in the left panels in Figures 4.2A and 4.2B. Western blots of cell lysates were probed with anti-LysRS (Figures 4.2A and 4.2B, top panels), anti-Myc (middle panels), or anti-β-actin (bottom panels). Anti-LysRS detects both endogenous and exogenous LysRS, while anti-Myc detects only exogenous wild type and mutant forms of LysRS. The ratios of mutant LysRS/β-actin are similar for expression of most mutant LysRS constructs, but are less than the wild-type LysRS/β-actin ratio, which was set at 1.

In the cell lysate of both transfected and non-transfected cells, there is also a less abundant protein species staining with anti-LysRS that has a lower molecular weight (M_r= 62-63 kDa) than the endogenous LysRS (M_r =68 kDa). The smaller molecular weight species also appears in the viral lysate, where its

abundance relative to the full-length 68 kDa LysRS band has increased, as previously reported (32). The source of this species (proteolytic processing of full-length LysRS, or translation from an alternatively spliced mRNA), is not yet known. The overexpression of wild-type LysRS from an exogenous plasmid (fifth lane in the upper two panels in Figure 4.1A), which results in a LysRS species larger than the endogenous wild-type species since it contains a 20 amino acid N- terminal Myc tag, does not seem to increase the relative abundance of the 62-63 kDa LysRS species in the cytoplasm.

The incorporation of the LysRS variants into virions is shown on the right side of Figure 4.2A and 4.2B, which show Western blots of viral lysates probed with anti-LysRS (top), anti-Myc (middle), and anti-Capsid (bottom). Deletion of the N-terminal 207 amino acids does not affect the ability of LysRS to be incorporated into Gag VLPs, while deletion of the C-terminal amino acids (207-597) inhibits LysRS packaging. The anti-LysRS used in the top panel does show a small amount of Δ207-597 incorporated into the virus, while anti-Myc (middle panel) detects none of this species in the virion. The ratios listed at the bottom of the panel use the Myc-LysRS/Gag ratio because the anti-Myc is expected to show less variability in detecting the different deleted LysRS species than the anti-LysRS. Further mapping shown in Figure 4.2A and 4.2B reveals that a critical region in LysRS for incorporation lies between amino acids 249 and 309, i.e., C-terminal deletions not including this region do not affect packaging. Finer mapping shown in Figure 4.2B shows further that a critical region for LysRS incorporation lies between amino acids 249 and 260, i.e., C-terminal deletions of

LysRS up to and including amino acid 260 do not affect LysRS packaging, while LysRS with a C-terminal deletion up to and including amino acid 249 is not incorporated into Gag VLPs.

Taken together, the results shown in Figures 4.1 and 4.2 show that the *in vitro* interaction between Gag and LysRS is inhibited when the LysRS C-terminal deletion includes the sequence between amino acids 249 and 309, while the packaging of LysRS into Gag VLPs is inhibited when the C-terminal deletion of LysRS includes amino acids 249-260. The similarity of results obtained *in vitro* and *in vivo* indicates that the interaction between Gag and LysRS *in vivo* is likely to be a direct one.

4.5.3 Interaction of mutant and wild-type Gag with wild-type LysRS in vitro and in vivo— Figure 4.3A shows N- and C-terminal Gag deletion mutants, which were constructed and expressed in *E. coli*. The *E. coli* lysates were adsorbed to glutathione-agarose beads, and after washing as described above, incubated with recombinant wild-type His_6-LysRS (190). After removing unbound LysRS, the beads were resuspended directly in SDS sample buffer, boiled, and subjected to SDS-PAGE. Western blots were probed with either anti-LysRS or anti-GST. Figure 3A shows the relative amount of LysRS bound to mutant Gag for each of the constructs tested, where the LysRS/Gag ratio for wild-type Gag is given a value of 1.00. The Western blot data supporting these results are shown in panels B and C. Panel B shows a Western blot of the gel probed with anti-Capsid. The first lane (GST) represents a control experiment performed with GST alone, while the other lanes show the wild-type and mutant

forms of Gag eluted from the beads. The multiple bands of GST-Gag seen is a common degradation problem seen when Gag is expressed in bacteria (for example, see (25)). We conclude from these results that all mutants are expressed. Panel C shows a Western blot of the same samples probed with anti-LysRS. This data shows that deletion of the N-terminal 307 amino acids of Gag does not affect its interaction with LysRS, whereas removal of an additional 30 amino acids (Δ 1-337) reduced the interaction to 44% of the wild-type binding level. Deletion of the next 41 amino acids (Δ 1-378) abolished Gag-LysRS binding. C-terminal deletions that included the p6 and nucleocapsid (NC) sequences did not affect the Gag/LysRS interaction. These results, therefore, indicate that the C-terminal third of the capsid region in Gag is important for the Gag/LysRS interaction.

We next examined the ability of LysRS to be packaged into Gag VLPs composed of mutant Gag species (Figure 4.4A). Cells were transfected with the plasmids coding for the following Gag constructs: wild-type Gag; ZWt-p6, in which the NC sequence had been replaced with a yeast leucine zipper domain (Z) to allow for protein/protein interactions; ZWt, in which both NC and p6 sequences were removed and NC has been replaced with Z; ΔZWt-p6, a Gag construct which contains only the signal for myristylation in the matrix protein, the C-terminal third domain of capsid, the p2 domain, the Z sequence replacing the NC domain, and the p6 domain. It has previously been shown that all of these mutants can efficiently form Gag VLPs (1). 293FT cells were transfected with the plasmids coding for wild-type or mutant Gag constructs, and the ability of the

Gag VLPs to package LysRS was assessed by Western blots of VLP lysates probed with anti-Capsid (Figure 4.4B, left) or anti-LysRS (Figure 4.4B, right). The Western blot data shown in panel 4C indicate that all mutants retain the ability to package LysRS. The results indicate that the C-terminal third of capsid and/or the p2 domain may be involved in binding LysRS, which is supported by the *in vitro* binding data obtained (Figure 4.3). However, since the *in vitro* interactions shown in Figure 4.3C indicate that the Gag deletion mutant Δ363-500, which lacks p2, still binds to LysRS, p2 is clearly not involved in binding LysRS.

To determine which domain of Gag was responsible for interaction with LysRS in the cytoplasm of 293FT cells, the latter were transfected with plasmids coding for wild-type Gag and some of the mutant Gag constructs. The interaction was tested by immunoprecipitation with anti-LysRS, and the results are shown in the Western blots presented in Figure 4.5. Figure 4.5A shows the total expression of the Gag constructs in the cytoplasm, using β-actin as a reference, while Figure 4.5B shows the ability of anti-LysRS to immunoprecipitate the Gag species. Wt-Gag, ZWt (Figure 4.4A) and Δ363-500 (Figure 4.3A) interact with cellular LysRS, but Δ323-500 does not. Referring to Figure 4.3A, this data supports an interaction between amino acids 323-363 of capsid and LysRS, and shows that the p2 region is not critical for Gag-LysRS association, which is consistent with *in vitro* binding data (Figure 4.3C).

4.6 Discussion

Terminal deletion mutations in both HIV-1 Gag and human LysRS have been used to map interacting sites both *in vitro* and *in vivo*. *In vitro*, wild-type and terminally deleted forms of GST-LysRS or GST-Gag were bound to glutathione agarose, and their capability to bind to wild-type Gag or LysRS, respectively, was monitored (Figures 4.1 and 4.3). *In vivo*, 293FT cells were transfected with plasmids coding for deletion mutants of either LysRS (Figures 4.2A and 4.2B) or Gag (Figures 4.4 and 4.5), and the interaction between these proteins was monitored either by measuring the ability of LysRS to be incorporated into extracellular Gag VLPs, or by co-immunoprecipitation using anti-LysRS antibodies. Importantly, the same sequences were determined to be essential for Gag /LysRS binding by all methods of analysis used.

The importance of amino acids 308-362 at the C-terminus of capsid for interaction with LysRS *in vitro* was suggested by the deletion analysis shown in Figure 3. N-terminal deletions in Gag up to and including amino acid 307, and C-terminal deletions in Gag extending to amino acid 363, do not affect the *in vitro* interaction between Gag and wild-type LysRS. This conclusion is supported by *in vivo* work showing that removal of all of matrix (except the first 8 N-terminal amino acids, so as to maintain the myristylation site), the N-terminal two-thirds of capsid, all of p6, and replacement of NC sequences with the yeast leucine zipper domain (Z), still allows for the formation of Gag VLPs containing LysRS (Figure 4). The co-immunoprecipitation data shown in Figure 4.5, further help to narrow down the critical region for LysRS interaction to amino acids 323-362. Based

upon both the three dimensional structure of the C-terminal part of the HIV-1 capsid region (88), and *in vitro* analysis of mutations in this region (25, 88), the sequences in Gag that we have determined are critical for interaction with LysRS are part of the capsid dimer interface. The biological significance of this dimer interface in HIV-1 assembly is not clear, since this interaction is much weaker than the NC/NC interactions, which, facilitated by NC/RNA interaction, are probably the driving force in Gag oligomerization (25, 88). Nevertheless, this region, which can facilitate homodimerization between capsid molecules, is also important for interaction with LysRS, possibly through formation of a heterodimer.

Our results also support a role in Gag interaction for LysRS amino acid sequences within motif 1, a domain known to be critical for dimerization of class II aminoacyl-tRNA synthetases (28, 52, 66, 169). In particular, the importance of amino acids 208-259 in LysRS was shown by the fact that the N-terminal deletion of residues 1-207 (Figure 4.2A), and the C-terminal deletion of residues 260-597 (Figure 4.2B), does not affect packaging of LysRS into Gag VLPs, whereas constructs with deletions that include residues 208-259 abolish packaging (Figures 4.2A and 4.2B). While no N-terminal LysRS deletion construct was tested for its interaction with Gag *in vitro*, the fact that the LysRS deletion mutant Δ309-597 interacts with Gag, while the LysRS deletion mutant Δ249-597 does not (Figure 4.1), supports the conclusions derived from *in vivo* data.

As described in the introduction, eukaryotic LysRS is a class IIb synthetase with a distribution of functional domains shown in cartoon form in

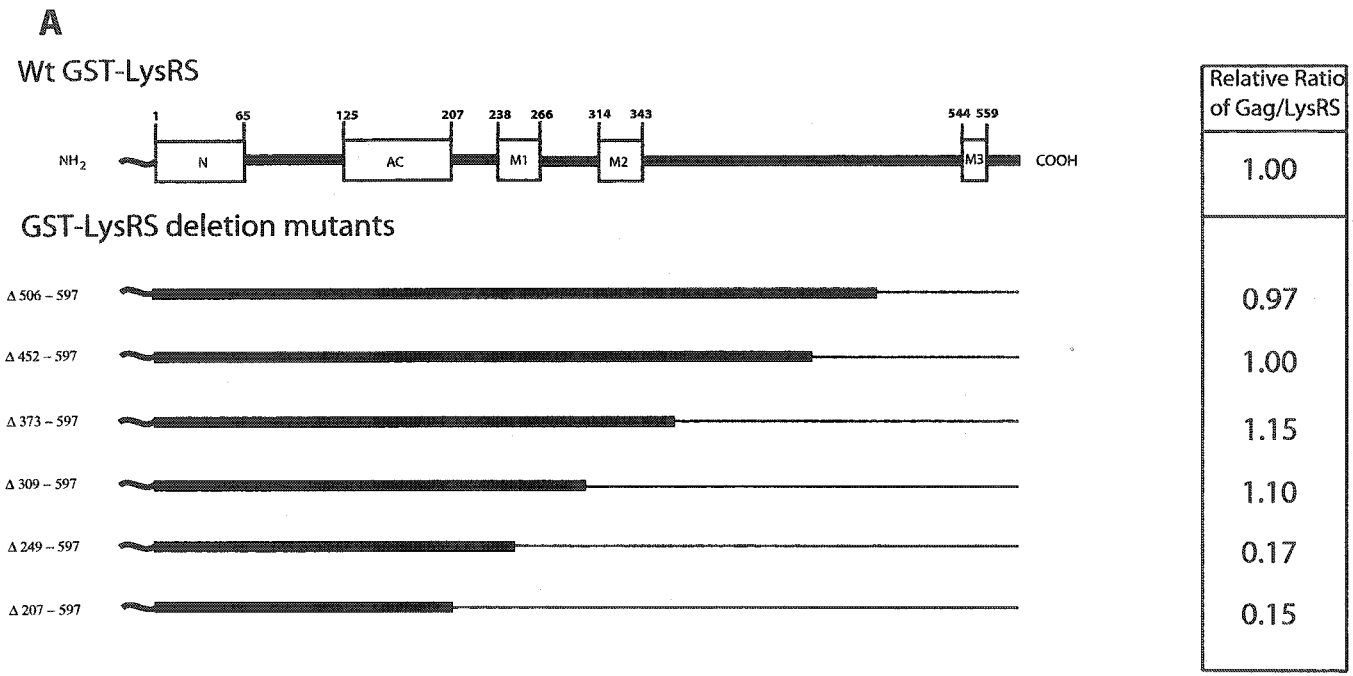
Figure 4.1A. Thus, the N-terminal deletion of residues 1-207 removes regions that are important for binding to $tRNA^{Lys}$, including the N-terminal extension and the anticodon binding domain (Figure 4.1), yet this construct can still interact with Gag (Figure 2A). The C-terminal deletion of residues 260-597 removes motif 2 and 3 sequences that constitute the catalytic domain of LysRS. These regions are also dispensable for Gag interaction (Figure 2B). A multiple sequence alignment of LysRSs from different species showed that the aminoacylation catalytic domain is highly conserved in this enzyme (190). Our results, therefore, suggest that the interaction of LysRS with Gag occurs independently of its ability to bind strongly to and aminoacylate $tRNA^{Lys}$. This supports earlier findings that LysRS is packaged into Gag VLPs or mutants virions independently of $tRNA^{Lys}$ packaging (32). Thus, we have previously shown that Gag VLPs efficiently package viral genomic RNA (151) and LysRS (32), but do not package $tRNA^{Lys}$, which requires the additional presence of Gag-Pol (151). Gag-Pol is presumably required to stabilize the presence of $tRNA^{Lys}$ in the Gag/Gag-Pol/$tRNA^{Lys}$/LysRS packaging complex.

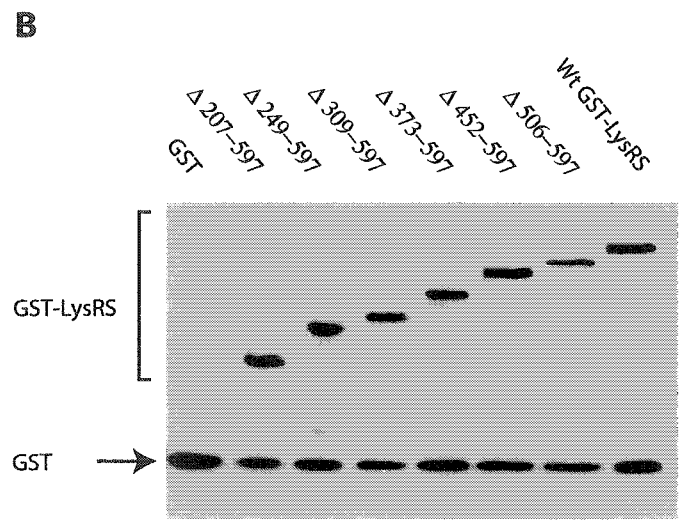
In contrast to LysRS packaging, which can occur independently of $tRNA^{Lys}$ packaging, we previously showed that the latter was directly correlated with LysRS interaction (119). In particular, tRNA anticodon mutants that were poorly aminoacylated were also not efficiently packaged. Thus, tRNA packaging appears to depend upon productive interaction with LysRS, whereas LysRS packaging depends only on interaction with Gag.

In conclusion, our data show that residues responsible for the homodimerization of capsid and LysRS are also critical for facilitating the interaction between these two molecules. This suggests that the interaction between LysRS and Gag involves heterodimer formation using the same interface used by each molecule for homodimerization. This implies that LysRS may be incorporated into the virion as a monomer. The effect of this interaction upon the multimerization of the Gag molecule would be more difficult to predict since putative regions of interactions between Gag molecules have been identified as occurring at multiple sites in the C-terminal half of Gag, and include the C-terminal half of capsid (17, 88, 141, 165), the p2 spacer region (1, 132, 168), nucleocapsid (25, 26, 47, 185), and p6 (90). Also, the virus is composed of approximately 1500 Gag molecules (197), and it is very probable that only a small fraction of these are involved in the interaction with LysRS.

Alternatively, homodimers of LysRS and Gag may be required in the LysRS/Gag interaction to allow sequences elsewhere in these molecules to participate in this interaction. This is less likely since the experiments performed herein, *in vitro* and *in vivo*, indicate that sequences deleted both upstream or downstream of the dimerization sites in LysRS and in Gag are not required for formation of the Gag /LysRS complex.

Figure 4.1. **In vitro interaction between wild-type Gag and wild-type or mutant LysRS**. Wild-type and C-terminal-deleted LysRS, which are N-terminal tagged with GST, were expressed in *E. coli*. The *E. coli* lysates were adsorbed to glutathione-agarose beads, and after washing, the beads were incubated in binding buffer containing 3 ug purified recombinant HIV-1 Gag. After further washes, beads were resuspended directly in SDS sample buffer, boiled, and subjected to SDS-PAGE. Western blots of the eluted material were probed with either anti-GST (panel B) or anti-Capsid (panel C). Panel A shows the wild-type and mutant LysRS variants tested. The cartoon at the top shows the various LysRS domains and the amino acid positions (numbers) at which they occur. The unnumbered N-terminal squiggle represents glutathione-S-transferase. Amino acid sequences deleted are shown graphically as thin lines, and are listed to the left of each mutant. N, N terminal domain; AC, anticodon binding domain; motifs 1, 2, and 3, are sequence elements characteristic of class II tRNA synthetases, and are associated with functions described in the text. The right side of panel A lists the relative amount of Gag bound to mutant LysRS, where the Gag/LysRS ratio for wild-type LysRS is given a value of 1.00. These values were obtained from the Western blot data shown in panels B and C, using UN-SCAN-IT gel™ automated digitizing system.


A
Wt GST-LysRS
1
65
125
207
238
266
314
343
544
559
NH2
N
AC
M1
M2
M3
COOH
GST-LysRS deletion mutants
Δ 506 – 597
Δ 452 – 597
Δ 373 – 597
Δ 309 – 597
Δ 249 – 597
Δ 207 – 597
Relative Ratio of Gag/LysRS
1.00
0.97
1.00
1.15
1.10
0.17
0.15


B
GST
Δ 207–597
Δ 249–597
Δ 309–597
Δ 373–597
Δ 452–597
Δ 506–597
Wt GST-LysRS
GST-LysRS
GST

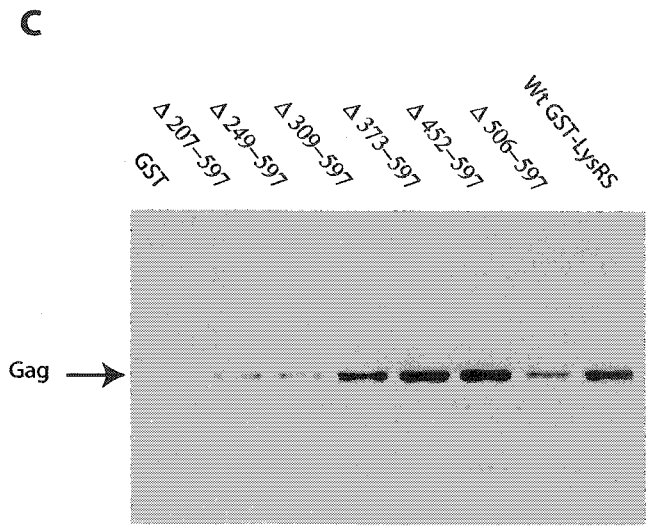
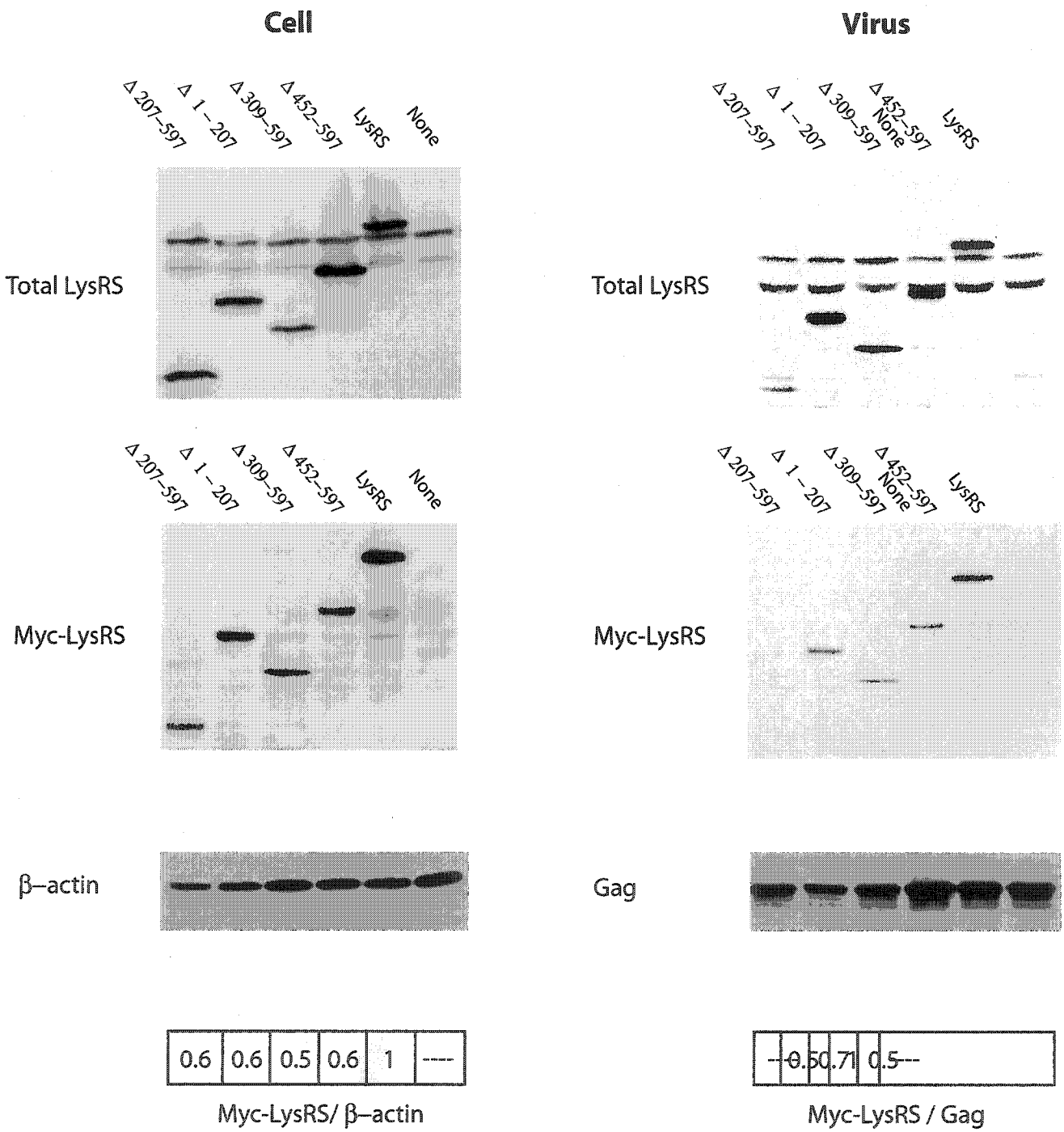

C
GST
Δ 207–597
Δ 249–597
Δ 309–597
Δ 373–597
Δ 452–597
Δ 506–597
Wt GST-LysRS
Gag

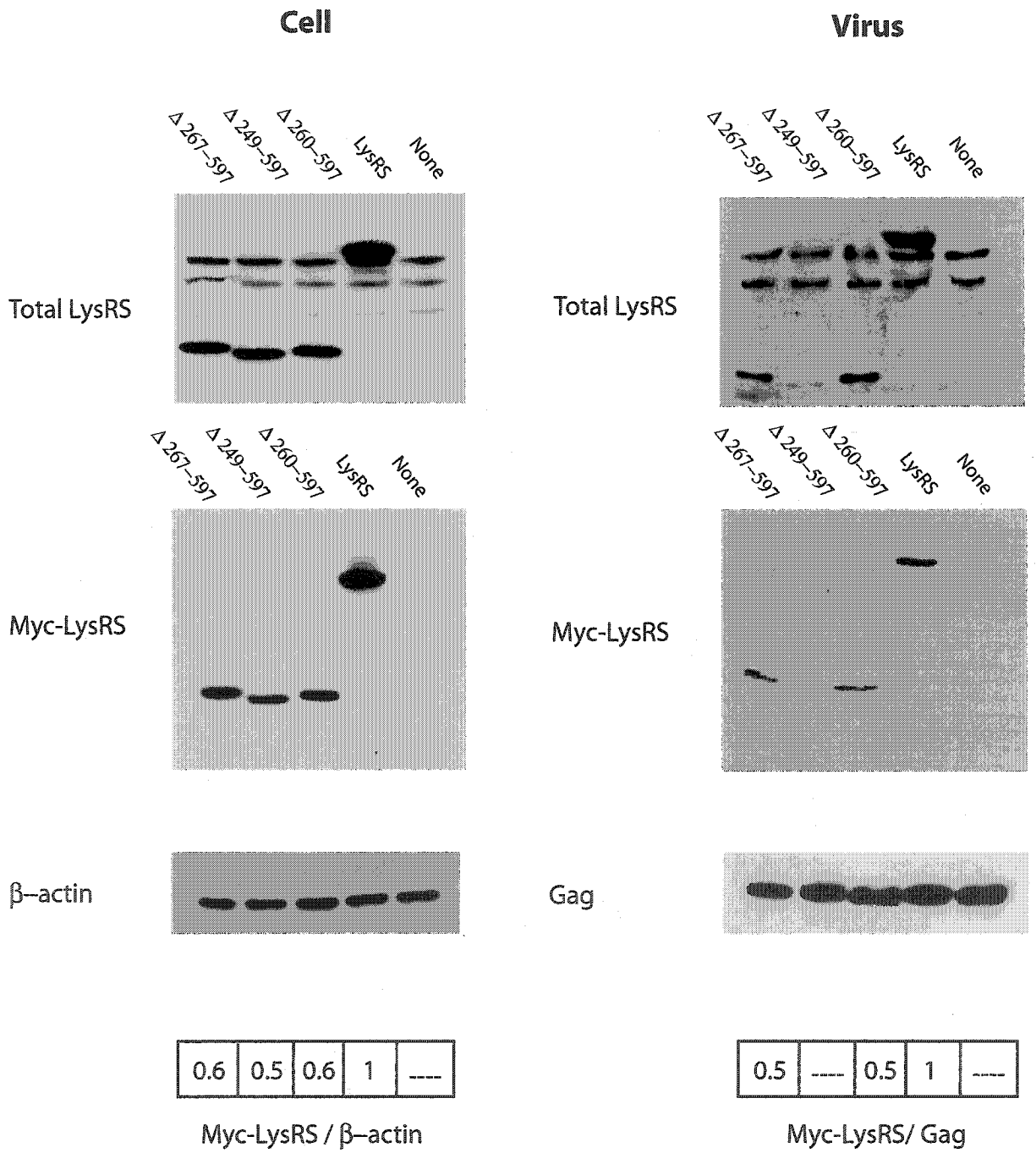
Figure 4.2. **The ability of mutant LysRS to be incorporated into Gag viral-like particles (VLPs).** Plasmids coding for N- and C-terminal LysRS deletion mutants were cotransfected into 293FT cells with a plasmid containing HIV-1 proviral DNA. The LysRS produced contained C-terminal Myc sequences. The designation of sequences deleted for each mutant uses amino acid numbers corresponding to those shown at the top of Figure 4.1A. The left side of panels A and B show Western blots of lysates of cells cotransfected with HIV-1 proviral DNA and LysRS mutants. The blots are probed, respectively, with anti-LysRS, anti-Myc, and anti-β-actin. Anti-LysRS detects both endogenous and exogenous LysRS, while anti-Myc detects only exogenous LysRS. The expression of exogenous LysRS, listed as the Myc-LysRS/β-actin ratio, is shown at the bottom of the blots. The right side of panels A and B show Western blots of lysates of viruses produced from the cells probed, respectively, with anti-LysRS, anti-Myc, and anti-Capsid. The incorporation of exogenous LysRS is listed as the Myc-LysRS/ Gag ratio at the bottom of the blots.

A


Cell
Virus
Δ207–597
Δ1–207
Δ309–597
Δ452–597
LysRS
None
Total LysRS
Myc-LysRS
β–actin
Gag
0.6
0.6
0.5
0.6
1

0.5
0.7
0.5
Myc-LysRS/ β–actin
Myc-LysRS / Gag

B


Cell
Virus
Δ267–597
Δ249–597
Δ260–597
LysRS
None
Total LysRS
Myc-LysRS
β–actin
Gag
0.6
0.5
0.6
1

0.5

0.5
1

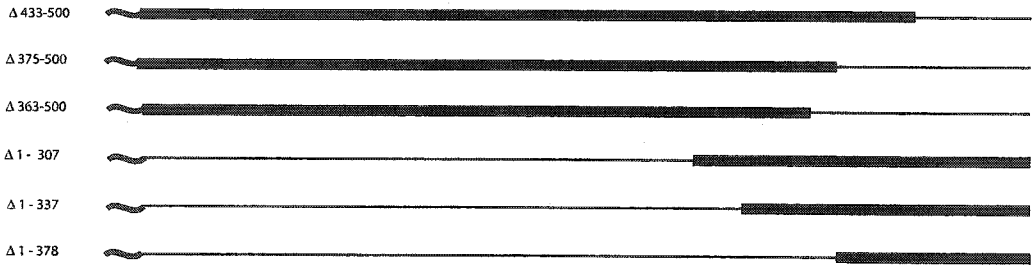
Myc-LysRS / β–actin
Myc-LysRS/ Gag

Figure 4.3 **In vitro interaction between wild-type LysRS and wild-type or mutant Gag**. Wild-type and N- or C-terminal-deleted Gag, which was N-terminally tagged with GST, were expressed in E. coli. The E. coli lysates were adsorbed to glutathione-agarose beads, and after washing, the beads were incubated in binding buffer containing 4 ug purified recombinant His_6-LysRS. After further washes, beads were resuspended directly in SDS sample buffer, boiled, and subjected to SDS-PAGE. Western blots of the eluted material were probed with either anti-GST (panel B) or anti-LysRS (panel C). Panel A shows the wild-type and mutant Gag variants tested. The cartoon at the top shows the various Gag domains and the amino acid positions (numbers) at which they occur. The unnumbered N-terminal squiggle represents glutathione-S-transferase. Amino acid sequences deleted are shown graphically as thin lines, and are listed to the left of each mutant. MA, matrix domain; CA, capsid domain; NC, nucleocapsid; p6, p6 domain. The right side of panel A lists the relative amount of wild-type LysRS bound to wild-type or mutant Gag, where the LysRS/Gag ratio for wild-type Gag is given a value of 1.00. These values were obtained from the Western blot data shown in panels B and C.

A

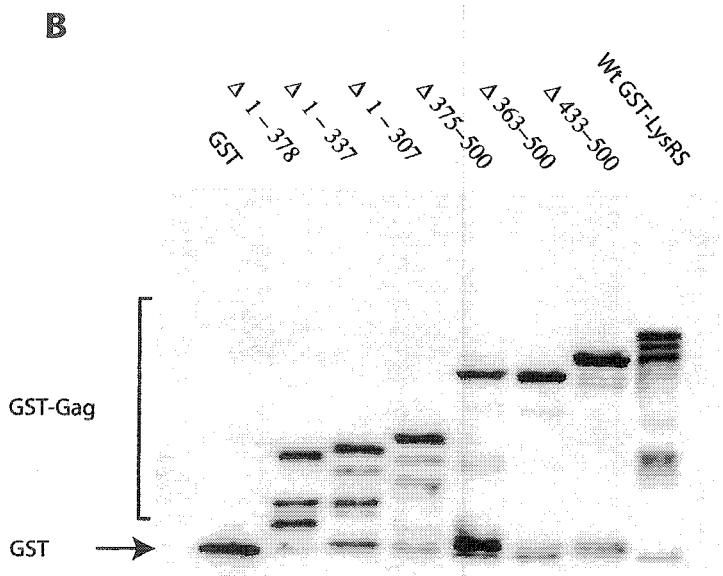
Wt GST-Gag

GST-Gag deletion mutants



Relative Ratio of LsyRS/Gag
1.00
0.80
1.00
0.90
1.00
0.44

B



C

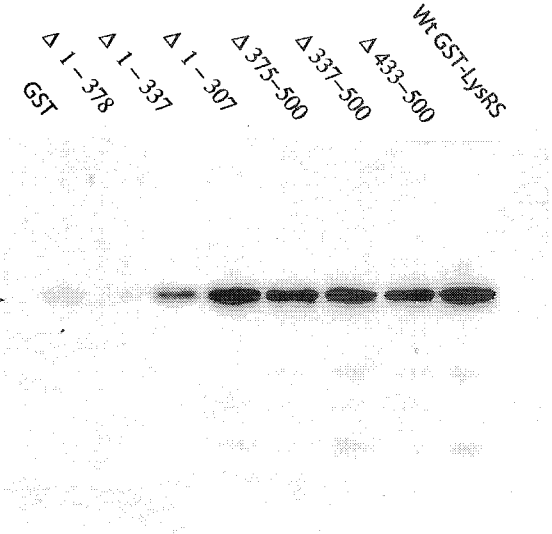


Figure 4.4. **The ability of endogenous LysRS to be incorporated into wild-type or mutant Gag viral-like particles (VLPs).** Plasmids coding for wild type or mutant Gag were transfected into 293FT cells. The Gag deletion mutants are shown in panel A, and their construction and has been previously described (1). Wt-Gag, wild-type Gag. ZWt, a construct in which the NC, p1, and p6 domains have been deleted, and NC has been replaced with a yeast leucine zipper domain, Z. ZWt-p6, a construct in which the p6 domain has been added back to ZWt. ΔZWt-p6, a construct in which the deletion includes all but the first 8 amino acids of MA, approximately two-thirds of capsid, as well as replacement of the entire NC domain with the Z domain. Western blots of lysates of Gag VLPs produced from cells transfected with the different Gag plasmids and probed with either anti-Capsid or anti-LysRS are shown in panels B and C, respectively. While monoclonal antibodies that are specifically reactive with HIV-1 capsid (Zepto Metrocs, Inc) were used for the upper gel in panel B, a different monoclonal antibody to C-terminal of HIV-1 capsid, was required to detect ΔZ-Wt-p6 (NIH AIDS Research and Reference Reagent Program).

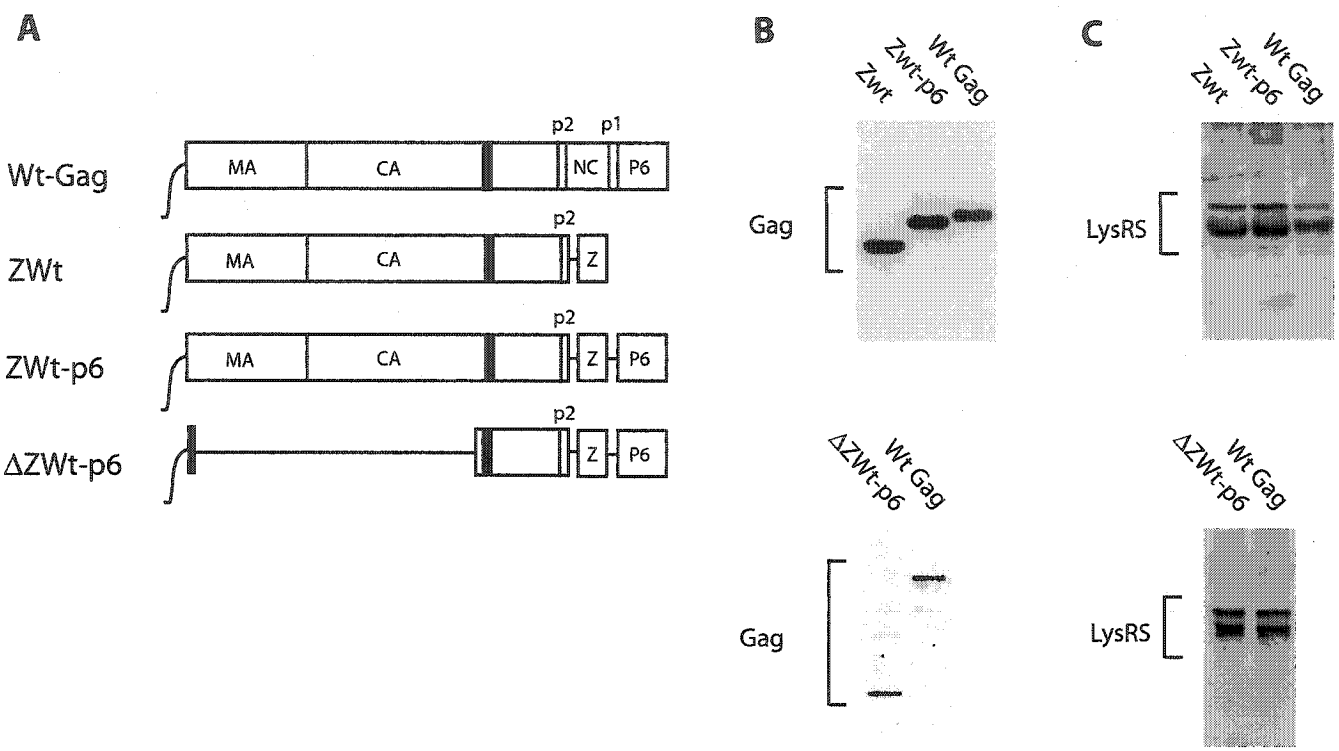
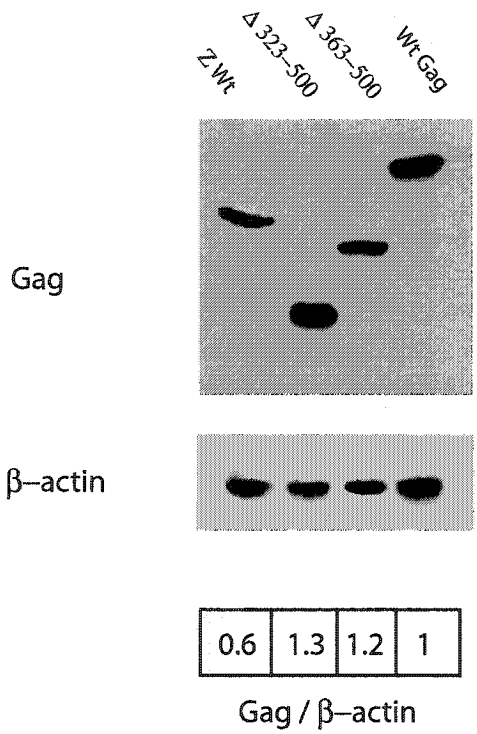

A
Wt-Gag
MA
CA
p2
NC
p1
P6
ZWt
Z
ZWt-p6
ΔZWt-p6
B
Wt Gag
Zwt-p6
Zwt
Gag
ΔZWt-p6
C
LysRS

Figure 4.5. **Interaction of endogenous LysRS with wild-type or mutant Gag in the cytoplasm of cells transfected with plasmids coding for Gag variants.** Interaction was measured by the ability to immunoprecipitate both Gag and LysRS with anti-LysRS. A. Western blot of lysates of transfected cells, probed with anti-Capsid (top) or anti-β-actin (bottom), respectively. The Gag/β-actin ratio obtained from quantitation of the gel data is listed below the blots. B. Western blot of anti-LysRS immunoprecipitates from lysates of Gag VLPs probed with anti- Capsid.

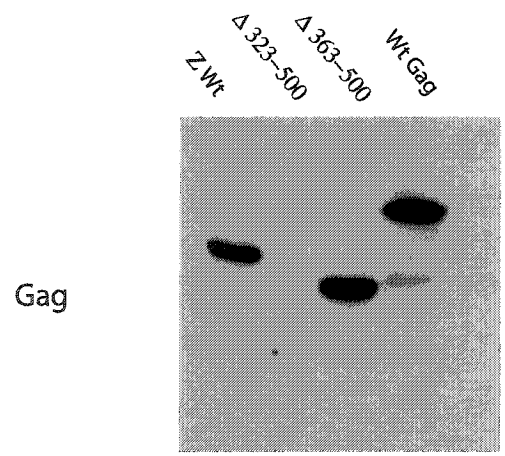
A



0.6	1.3	1.2	1

Gag / β–actin

B



Chapter 5

General Discussion

During HIV-1 assembly, $tRNA^{Lys}$ isoacceptors are selectively incorporated into HIV-1, and viral infectivity is related to the content of $tRNA^{Lys}$ in the viral population (82). We have shown previously that $tRNA^{Lys}$ packaging is dependent on the incorporation of the viral precursor Gag-Pol, and independent of viral genomic RNA incorporation (151). However it has not been clear what signals target $tRNA^{Lys}$ for packaging into HIV-1. In the cytoplasm, $tRNA^{Lys}$ is bound to tRNA binding proteins such as lysyl-tRNA synthetase (LysRS), and we have recently discovered that LysRS specifically incorporates into HIV-1 (32). Therefore LysRS is a prime candidate to act as the signal, which targets $tRNA^{Lys}$ for incorporation into HIV-1.

In this thesis, we have examined the parameters influencing incorporation of lysyl-tRNA synthetase into HIV-1, and the role of LysRS in selective packaging of $tRNA^{Lys3}$ in HIV-1. In chapter 2, we showed that the ability of $tRNA^{Lys}$ to interact with LysRS was required for the tRNAs incorporation into the virus, while chapter 3 showed that the ability of LysRS to aminoacylate tRNA was not required for $tRNA^{Lys}$ packaging. In chapter 4, we mapped the interacting sites between LysRS and Gag.

In Chapter 2, we constructed $tRNA^{Lys3}$ anticodon mutants, and examined whether the ability of these mutants to bind to LysRS (measured by their state of aminoacylation) was correlated with their ability to be incorporated into HIV-1. Our results indicated that there is a direct correlation between the aminoacylation state of these $tRNA^{Lys3}$ mutants and their incorporation into HIV-1. Since the $tRNA^{Lys3}$ anticodon is required for interaction with LysRS, it was not clear from

these results if the ability of LysRS to aminoacylate $tRNA^{Lys}$ is also required for $tRNA^{Lys}$ incorporation into viruses. This was further examined, in chapter 3, where we constructed two mutant LysRS species, one of which had lost its ability to aminoacylate $tRNA^{Lys3}$, and the other of which has lost its ability to interact with $tRNA^{Lys3}$. We examined the effect of these mutant LysRS species upon $tRNA^{Lys}$ incorporation into HIV-1, and found that the $tRNA^{Lys}$ binding site on LysRS rather than the catalytic site, is crucial for selective packaging of $tRNA^{Lys3}$.

Other studies from our laboratory also support a role for LysRS in promoting $tRNA^{Lys}$ incorporation into virus. For instance, in one study (83), we have shown that LysRS appears to be a limiting factor for the amount of $tRNA^{Lys}$ packaged into the virion. HIV-1 contains approximately 20 molecules of $tRNA^{Lys,}$ averaging 12 molecules of $tRNA^{Lys1,2}$ and 8 molecules of $tRNA^{Lys3.}$ Expressing an exogenous $tRNA^{Lys3}$ gene can double the number of $tRNA^{Lys3}$ molecule in the virion, but the number of $tRNA^{Lys1,2}$ molecules/virion decreases accordingly, maintaining the same number of total $tRNA^{Lys}$ / virion. There is therefore, a factor that limits the number of $tRNA^{Lys}$ molecules packaged per virion. When cytoplasmic LysRS is increase by co-transfecting cells with plasmids coding for exogenous LysRS or HIV-1 proviral DNA, more LysRS is packaged into the virus, and the viral $tRNA^{Lys}$ content doubles, with incorporation of all $tRNA^{Lys}$ isoacceptors increasing. The Gag-Pol:Gag ratio does not change during this process, indicating that the binding sites for the $tRNA^{Lys}$/ LysRS on the Gag/Gag-Pol complexes are unsaturated. Therefore the amount of total $tRNA^{Lys}$ incorporated into virion is directly related to the amount of LysRS package into

HIV-1, which suggests LysRS is the limiting factor for incorporation of $tRNA^{Lys}$. Collectively, our results, supports a model for the selective packaging of $tRNA^{Lys3}$ into HIV-1 in which a $tRNA^{Lys}$/LysRS complex interacts with a Gag/Gag-Pol complex, and figure 1.6 illustrates this model. In this model, we propose that Gag interacts with LysRS, and Gag-Pol interacts with $tRNA^{Lys}$

In chapter 4, we mapped the binding sites involved in the LysRS/Gag interaction, to the LysRS amino acids sequences including motif 1 of LysRS and C-terminal 54 amino acids of CA within Gag. Both motif 1 and the sequences within C-terminal of capsid participate in homodimerization of LysRS (181) and CA (62), respectively. The sequences flanking these regions in both LysRS and Gag can be deleted without effecting the incorporation of LysRS into VLPs.

The incorporation into Gag VLPs of LysRS missing tRNA binding domain is further evidence that LysRS incorporation occurs independently of $tRNA^{Lys}$ incorporation. There are still many unanswered questions about where in the cells LysRS and Gag interact. There are approximately 25 LysRS molecules per virion (30), while each virion contains approximately 1500-2000 Gag molecules (89). It is not clear if the low concentration of LysRS in the virus is due to competition between the Gag/Gag and Gag/LysRS interaction, since the major sequences involved in the Gag/Gag interaction, might not exclude LysRS (216). Alternatively, one might consider the limited LysRS reservoir that is available to Gag in the cytoplasm. Most of the cytoplasmic LysRS is associated with the HMW aaRS (78) but this does not seem to be the cellular source of viral LysRS, First, other than LysRS (30), none of the members of this complex tested were

found in the virus (100). Second, p38 is a non-aaRS protein in the HMW aaRS complex that binds most tightly to LysRS (181), and it too is not incorporated into virus. Third, the initiation of formation of the HMW aaRS complex requires the p38/LysRS interaction (181), and LysRS mutants not able to bind to p38 are still able to be packaged into viruses. There must be, therefore another source of cytoplasmic LysRS available to Gag.

Dr. Guo in our laboratory has recently discovered that the concentration of LysRS in the virion is related to the level of newly-synthesized, rather than total LysRS, in the cells. This suggests that LysRS interacts with Gag before it becomes part of the HMW aaRS complex in the cytoplasm. It is not known at which cellular site this occurs, especially since both LysRS and Gag have been detected in both the nucleus and the cytoplasm.

Other retroviruse families may also use the aminoacyl-tRNA synthetases as the signal to package their cognate primer tRNA into viruses. Thus, our laboratory has shown that Rouse sarcoma virus (RSV), which uses $tRNA^{Trp}$ as a primer, specifically packages TrpRS (30). Among different families of retroviruses, the diversity of Gag proteins could explain the incorporation of different aaRSs into retroviruses. It would be interesting to construct chimera Gag molecules, which contain Gag sequences from different retroviruses. The chimeric Gag might package alternate aaRS to the cognate primer tRNA. This would allow us to better understand the Gag/aaRS interaction.

The LysRS and Gag binding domains could serve as novel targets for pharmaceutical anti-HIV drug design. We have already shown that we can

transfect cells with HIV-1 proviral DNA and siRNA specific for LysRS, to study the effects of diminished cellular LysRS upon $tRNA^{Lys}$ packaging, $tRNA^{Lys}$ annealing to viral genomic RNA, and viral infectivity. Virus produced from cells that were transfected with siRNA show reduced $tRNA^{Lys}$ packaging, reduced $tRNA^{Lys}$ annealing to viral genomic RNA, and reduced viral infectivity (95). Molecules that can inhibit Gag/LysRS interaction might therefore be used one day as inhibitors of HIV-1 replication.

Chapter 6

Contributions to Original knowledge

The following is a summary of my contributions toward an understanding of retroviral replication during my Ph.D studies in Dr. Kleiman's lab.

Chapter 1:

In chapter 1, we have shown for the first time that $tRNA^{Lys3}$ anticodon is the major determinant for selective packaging of $tRNA^{Lys3}$. Using $tRNA^{Lys3}$ aminoacylation to measure the interaction between LysRS and wild-type and mutant $tRNA^{Lys3}$, we showed that there is a direct correlation between the ability of a tRNA to be aminoacylated (interact with LysRS) and its ability to be packaged into HIV-1. We also showed for the first time that U35 and U36 bases within the $tRNA^{Lys3}$ anticodon domain are of great importance for this interaction.

Chapter 2

In chapter 2, we showed that while the $tRNA^{Lys3}$/LysRS interaction is crucial for selective packaging of $tRNA^{Lys}$, the ability of LysRS to aminoacylate $tRNA^{Lys3}$ is not required for its incorporation into HIV-1.

Chapter 3

For the first time, we demonstrated *in vitro* that there is a direct interaction between Gag and LysRS. We have also mapped the binding sites within these

molecules using both *in vivo* and *in vitro* techniques. We found that sequences which include motif 1 of LysRS and the C-terminal region of capsid are responsible for the Gag/LysRS interaction.

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