Role and Regulation of tRNA^{Lys3} in the life cycle of Human Immunodeficiency Virus Type 1

by

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To my parents, Ivan and Ivanka Gabor, and to my husband Paul Kasnakian, for their love, understanding, encouragement and support.

ABSTRACT

$tRNA^{Lys3}$ is selectively packaged into HIV-1 during viral assembly, and is placed upon the primer binding site (PBS) of the viral genome where it acts as the primer for reverse transcriptase. This Ph.D. project involved studying the factors involved in the regulation of $tRNA^{Lys}$ packaging, as well as the effect of overexpression of $tRNA^{Lys3}$ in the virion.

Previous work from our laboratory has shown that both $tRNA^{Lys1,2}$ and $tRNA^{Lys3}$ are selectively packaged into HIV-1, at a rate of approximately 20 molecules per virus, with the ratio of $tRNA^{Lys1,2}$ to $tRNA^{Lys3}$ at approximately 12:8. Overexpression of cellular $tRNA^{Lys3}$ results in the near doubling of $tRNA^{Lys3}$ concentration with the virus, with a proportional decrease in $tRNA^{Lys1,2}$, all the while keeping the viral concentration of $tRNA^{Lys}$ constant. I have shown that an increase in viral $tRNA^{Lys3}$ concentration is accompanied by an increase in $tRNA^{Lys3}$ annealing to the PBS, and an increase in viral infectivity.

Studies from our laboratory have also shown that the viral precursor protein GagPol (precursor for reverse transcriptase, integrase and RNaseH) is required for $tRNA^{Lys}$ packaging. A $tRNA^{Lys}$-binding protein, lysyl tRNA synthetase (LysRS), has recently been shown to be selectively packaged into HIV-1, and requires the presence of Gag, but not GagPol, for its incorporation. I have demonstrated that when a LysRS gene is co-transfected with protease-negative HIV-1 proviral DNA into COS7 cells, the resulting virus packages almost twice as much $tRNA^{Lys}$ than virus without LysRS overexpression. Furthermore, I have shown that the ratio of GagPol:Gag does not change when the viral concentration of $tRNA^{Lys}$ is increased, indicating that GagPol is not a limiting factor in $tRNA^{Lys}$ packaging, and that LysRS is.

Overexpression of $tRNA^{Lys3}$, $tRNA^{Lys1,2}$, or LysRS also results in the stabilization of GagPol, which I showed by overexpressing either of these three molecules in COS7 cells also transfected with HIV-1 proviral DNA. Measurement of the ratios of viral reverse transcriptase (RT) or integase (IN) to the viral capsid

(CA) protein showed over two-fold increases in GagPol cleavage products (RT and IN) in these viruses when compared to wild-type. This increase could reflect an increase in cytoplasmic stabilization of GagPol, possibly during viral budding, and resulting in more GagPol being incorporated into the virion.

In another study related to the packaging of tRNA in HIV-1, I have found that another tRNA-synthetase and its cognate tRNA (AsnRS and $tRNA^{Asn}$) are packaged into HIV-1. As of yet, there is no known role for these two molecules in the viral life cycle. Since LysRS and AsnRS both belong to the same family of tRNA synthetases (Class 2b), it is possible that AsnRS molecules are mistakenly recognized as LysRS during viral packaging, and as a result of AsnRS's viral incorporation, $tRNA^{Asn}$ is also incorporated because of its cellular interactions with AsnRS. This interesting observation leads us to hypothesize that the select packaging of a tRNA into HIV-1 is facilitated by the select packaging of its cognate tRNA synthetase.

RÉSUMÉ

L'ARNt^{Lys3} subit un empaquetage sélectif dans le VIH-1 lors de l'assemblage viral, et il est placé sur le site d'amorce (PBS) du génome viral, où il amorce de la transcriptase inverse. Ce projet de 3^{e} cycle comprend l'étude des facteurs qui interviennent dans la régulation de l'empaquetage de l'ARNt^{Lys} et dans l'effet de la surexpression de l'ARNt^{Lys3} dans le virion.

Des études précédentes émanant de notre laboratoire ont déjà montré que l'ARNt^{Lys1,2} et l'ARNt^{Lys3} font l'objet d'un empaquetage sélectif dans le VIH-1 à raison d'environ 20 molécules par virus, le ratio de l'ARNt^{Lys1,2} à l'ARNt^{Lys3} étant d'environ 12:8. La surexpression de l'ARNt^{Lys3} cellulaire produit un quasi-dédoublement de la concentration d'ARNt^{Lys3} relativement au virus, accompagnée d'une diminution proportionnelle de l'ARNt^{Lys1,2}, alors que la concentration de l'ARNt^{Lys} reste constante. J'ai démontré qu'une augmentation de la concentration d'ARNt^{Lys3} viral s'accompagnait d'une diminution de l'ARNt^{Lys3} hybridé au niveau du PBS et d'une augmentation de l'infectivité virale.

Les études réalisées à notre laboratoire ont également montré que la protéine virale précurseur GagPol (précurseur de la transcriptase inverse, de l'intégrase et de la RNaseH) est essentielle à l'empaquetage de l'ARNt^{Lys}. Il a été récemment démontré qu'une protéine de liaison de l'ARNt^{Lys}, la lysyl-ARNt synthétase (LysRS), subit un empaquetage sélectif dans le VIH-1, et qu'elle nécessite la présence de Gag, mais non de GagPol, en vue de son incorporation. J'ai montré que lorsqu'un gène LysRS est co-transfecté avec de l'ADN proviral VIH-1 à protéase négative dans les cellules COS7, le virus qui en résulte contient presque deux fois plus d'ARNt^{Lys} qu'un virus dénué de surexpression LysRS. J'ai également montré que le ratio GagPol:Gag ne change pas lorsque la concentration virale de l'ARNt^{Lys} augmente, ce qui signifie que GagPol ne représente pas un facteur de limitation dans l'empaquetage de l'ARNt^{Lys}, contrairement à la LysRS.

La surexpression de l'ARNt^{Lys3}, de l'ARNt^{Lys1,2} ou de LysRS produit également la stabilisation de GagPol, ce qui j'ai démontré en ne surexprimant l'une ou l'autre de ces trois molécules dans les cellules COS7 transfectées d'ADN proviral du VIH-1. L'évaluation des ratios de transcriptase inverse virale (RT) ou intégrase (IN) dans la protéine capside virale (CA) dégage une multiplication par plus de deux des produits du clivage de GagPol (RT et IN) dans ces virus comparativement aux virus sauvages. Cette hausse pourrait refléter une augmentation de la stabilisation cytoplasmique de GagPol, peut-être pendant le bourgeonnement viral, causant une intégration accrue de GagPol dans le virion.

Dans une autre étude concernant l'empaquetage de l'ARNt dans le VIH-1, j'ai découvert qu'un autre ARNt synthétase et son ARNt parent (AsnRS et ARNt^{Asn}) sont présents dans l'empaquetage du VIH-1. À l'heure actuelle, on ne connaît pas le rôle de ces deux molécules dans le cycle de vie des virus. Étant donné que LysRS et AsnRS appartiennent toutes deux à la même famille des ARNt synthétases (Classe 2b), il est possible que les molécules AsnRS soient faussement reconnues comme des LysRS pendant l'empaquetage viral, et que cela entraîne l'incorporation virale d'AsnRS, l'ARNt^{Asn} étant alors incorporé à cause de ses interactions cellulaires avec AsnRS. Cette observation intéressante nous amène à poser l'hypothèse que l'empaquetage sélectif de l'ARNt dans le VIH-1 puisse être facilité par l'empaquetage sélectif de son ARNt synthétase parent.

PREFACE

This Ph.D. thesis was written in accordance with the Guidelines for Thesis Preparation from the Department of Graduate Studies and Research at McGill University. I have exercised the option of writing the thesis as a manuscript-based thesis. The guideline states: ".....Candidates have the option of including, as part of the thesis, the test of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the Thesis Preparation Guidelines with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.....The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting text that provide logical bridges between the different papers are mandatory.....The thesis must include the following: a table of contents; an abstract in English and French; an introduction which clearly states the rational and objectives of the research, a comprehensive review of the literature (in addition to that covered in the introduction to each paper); a final conclusion and summary; and, rather than individual reference lists after each chapter or paper, one comprehensive bibliography or reference list, at the end of the thesis, after the final conclusion and summary.....In general, when co-authored papers are included in a thesis, the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent....."

Chapter 1 presents an introduction and literature review of the research. A final general discussion is presented in chapter 5. The author's contribution to the original knowledge and references cited are listed at the end of the thesis. Three original papers, one published and two to be submitted for publication, have been integrated into this thesis. Connecting texts have been inserted between the chapters. The publications presented in the thesis are:

Chapter 2.
Gabor, J., Cen, S., Javanbakht, H., Niu, M., and L. Kleiman. 2002. Effect of altering the $tRNA^{Lys3}$ concentration in HIV-1 upon it annealing to viral RNA, GagPol Incorporation and viral infectivity. Journal of Virology. 76: 9096-9102.

Chapter 3.
Gabor, J., Cen, S., Niu, M., and L. Kleiman. 2003. Overexpression of $tRNA^{Lys}$ or lysyl tRNA synthetase results in an increase in reverse transcriptase and integrase in HIV-1. *Manuscript in preparation.*

Chapter 4.
Gabor, J., Halwani, R., Cen, S., Hirakata, M., and L. Kleiman. 2003. Identification of $tRNA^{Asn}$ and asparaginyl synthetase in HIV-1. *Manuscript in preparation.*

The candidate was responsible for all work described in this thesis, with the exception of the following work: part of tissue culture performed by Meijuan Niu in chapters 2 and 3, a primer extension assay in chapter 2 and a dot blot in chapter 3 by Dr. Shan Cen. The pLysRS.F plasmid in chapter 2 and 3 was provided by Dr. Shan Cen, as was the C-terminal His_6 tagged LysRS protein in chapter 4.

Other papers not included as part of this thesis, but with which the candidate was involved, are:

Perlman, M., Isel, C., Bec, G., Marquet, R., Gabor, J., Kleiman, L., and Kaufmann, G., 2002. Probing $tRNA^{Lys3}$ anticodon stem-loop conformation within HIV-1 priming complexes by anticodon nuclease. Manuscript in preparation.

Cen,S., Khorchid,A., Javanbakht,H., Gabor,J., Stello,T., Shiba,K., Musier-Forsyth,K., and Kleiman,L. Incorporation of lysyl tRNA synthetase into HIV-1. 2001. J.Virol. 75:5043-5048

Cen, S., Khorchid, A., Gabor, J., Rong. L., Wainberg, M.A., and Kleiman, L. 2000. Roles of $Pr55^{gag}$ and NCp7 in $tRNA^{Lys3}$ genomic placement and the initiation of reverse transcription in HIV-1. Journal of Virology 74: 10796-10800.

Huang, Y., Khorchid, A., Gabor, J., Wang, J., Li, X., Darlix, J-L., Wainberg, M.A., and Kleiman, L. 1998. The role of nucleocapsid and U5 stem/A-rich loop sequences in $tRNA^{Lys3}$ genomic placement and initiation of reverse transcription in HIV-1. Journal of Virology 72: 3907-3915.

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I would also like to thank Carolina Caro, Dimitri Motakis, Cathy Cammaleri, Pouya Pakneshan, Hassan Javanbakht, Elana Cherry and Sylvie Wise, all without whom my stay at the Lady Davis Institute would not have been half as enjoyable.

Finally, I wish to thank my family for their encouragement, understanding and support throughout my graduate studies, especially my husband Paul, for his love, encouragement and patience.

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TABLES AND FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AMV	Avian myeloblastosis virus
BLV	Bovine leukemia virus
CA	capsid protein
DIS	dimerization signal
DNA	deoxyribonucleic acid
EIAV	Equine infectious anemia virus
ENV	envelope protein
ER	endoplasmic reticulum
FeLV	Feline leukemia virus
HFV	Human foamy virus
HIV	Human immunodeficiency virus
HTLV	Human T-cell leukemia virus
IN	integrase protein
KDa	kilodalton
LAV	Lymphadenopathy-associated virus
LTR	long terminal repeat
LysRS	tRNA Lysyl synthetase
MA	matrix protein
MHR	major homology region
MMTV	Mouse mammary tumor virus
Mo-MULV	Moloney murine leukemia virus
MPMV	Mason-Pfizer monkey virus
NC	nucleocapsid protein
NLS	nuclear localization signal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	primer binding site
PCR	polymerase chain reaction
PPT	poly purine tract
PR	protease protein
RNA	ribonucleic acid
RNase	ribonuclease
RRE	Rev-response element
RSV	Rous sarcoma virus
RT	reverse transcriptase
SIV	Simian immunodeficiency virus
SU	surface protein
TAR	trans-activating region
TM	transmembrane protein
tRNA	transfer RNA
U3	3' unique region
U5	5' unique region

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The retroviruses

In 1911, Peyton Rous discovered that he was able to transmit malignant sarcoma from infected chickens into healthy chickens, using cell-free filtrates taken from tumors of the infected chickens. This finding led him to correctly hypothesize that the chicken filtrate contained a type of infectious particle (which later came to be termed "virus") that was responsible for the tumor development in the chickens (220). Since this discovery of the Rous sarcoma virus, retroviruses have been the topic of intense study, especially in the last two decades since the discovery of Human immunodeficiency virus, or HIV, which has proven to be the cause of one of the worst epidemics to affect mankind.

Most retroviruses identified to date have been found in vertebrate hosts. They are associated with many diseases, including neurological disorders, cancers, wasting diseases, and immunodeficiencies. Conversely, some retroviruses infect their host for life without any obvious negative effects upon the host.

The viruses that make up the Retroviridae Family are unique in many ways, with their most defining characteristic being their mechanism of replication. A controversial idea proposed by Howard Temin in the 1960's stated that genetic information could flow from RNA to DNA in retroviruses. This idea that went against the "Central Dogma" of genetics, a theory stating that the only direction that genetic information could flow was from DNA to RNA. Temin's idea was confirmed in 1970, both by Temin himself (253) and independently by David

Baltimore (9) with the discovery of reverse transcriptase (RT), the retroviral enzyme that transcribes RNA to DNA.

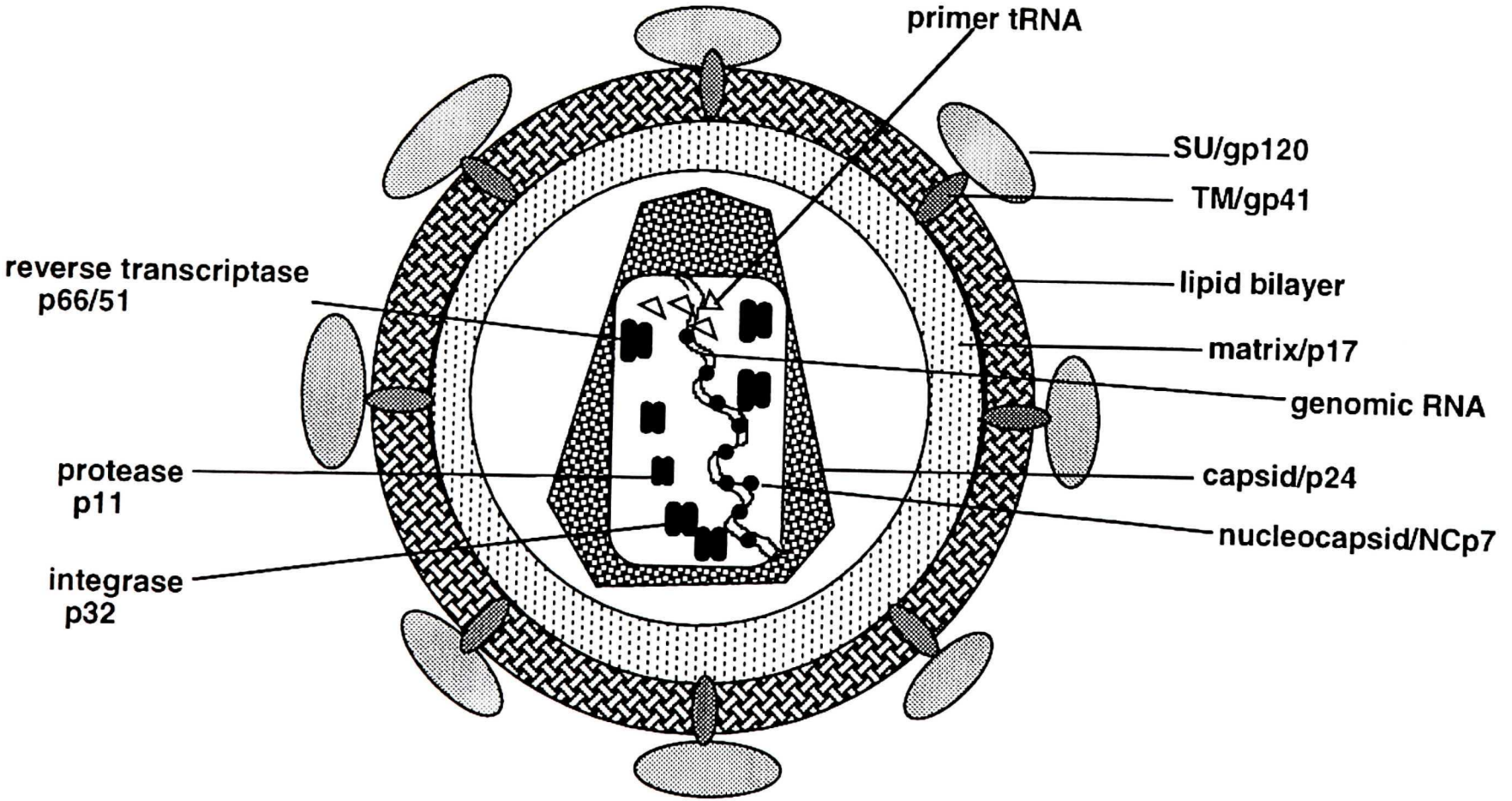
Another important characteristic of the retroviruses is their ability to insert the proviral DNA made by RT into host cell DNA. Consequences of this include the activation or inactivation of specific genes near the integration site, and a viral latency that can last for as long as 10 years until the activation of the provirus.

1.1.1 Virion Structure

Retroviruses are spherical and enveloped. They are approximately 80 to 100 nm in diameter, and the main structural components from outside to inside are the envelope, the core and the genome-nucleoprotein. The envelope contains glycosylated viral proteins that are products of the *env* gene, arranged in trimers. The core is composed of capsid (CA), a product of the *gag* gene. The core is circular in some retroviruses. In others, specifically in lentiviruses (i.e. HIV-1), it is cone or rod-shaped. Within core is the viral genomic RNA, which is associated with the nucleocapsid protein (NC), another products of the Gag protein, a host cell tRNA which is used as the primer for reverse transcriptase, and the viral enzymes reverse transcriptase (RT), integrase (IN), and protease (PR), which are all products of the GagPol precursor protein. These components are shown in Figure 1.1.

Figure 1.1. The HIV-1 virion.

Figure 1.1. The HIV-1 virion



1.1.2 Simple VS Complex Retroviruses

Retroviruses can be broadly divided into two categories based upon their genetic organization and regulation of gene expression: simple and complex (61), (62). All retroviruses, whether simple or complex, possess three genes that code for three basic viral precursor proteins: Gag, Pol and Env. In addition to these three genes, complex retroviruses contain additional genes that code for regulatory proteins, which are derived from multiply spliced mRNA's. Figure 1.2 shows examples of the genetic organization of both simple and complex viruses. All oncogenic retroviruses are classified as simple retroviruses, with the exception of the HTLV-BLV group. Complex retroviruses include HTLV-BLV, lentiviruses, and spumaviruses.

1.1.3 Virion Structure

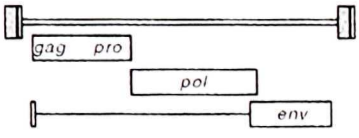
Retroviruses are also classified upon the basis of their morphological characteristics and subcellular distribution, which may reflect differences in modes of assembly (252). The four types of retrovirus morphology are type A, type B, type C and type D.

Type A particles are formed intracellularly, are spherical, and contain immature cores. They were first viewed as the immature intracellular form of Mouse mammary tumor virus (MMTV). Intracisternal A particles bud from intracellular membranes, and are often found in cells producing type C or type D particles, but without any known function. Intracytoplasmic forms of type A particles have been shown to be the precursor of some type B particles.

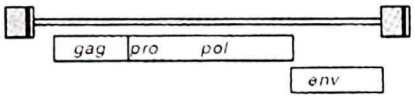
Figure 1.2. Genetic organization of simple and complex retroviruses. Adapted from (54).

Simple Retroviruses

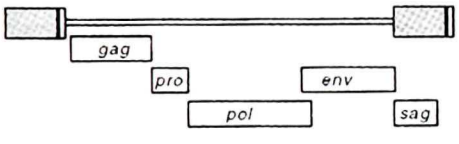
ALV



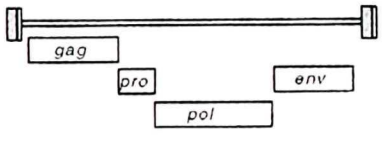
MLV



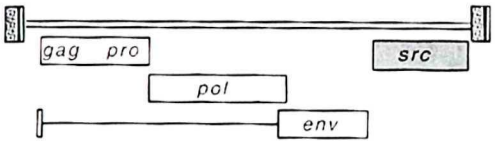
TV



MV

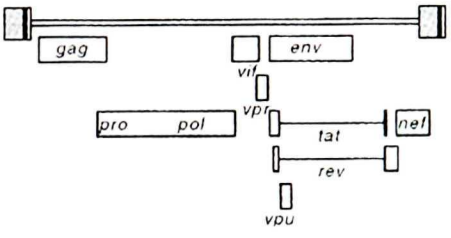


SV

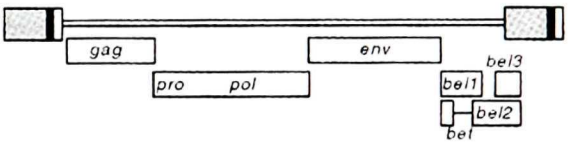


Complex Retroviruses

HIV



HFV



Type B particles are enveloped, extracellular viruses. Their immature forms resemble type A morphology, but they subsequently mature to form electron-dense cores, or nucleocapsids. Type B viruses also possess surface spikes extending outward from the envelope. Type B morphology is characteristic of MMTV viruses.

Type C viruses do not have complete intracellular forms, but form crescent-shaped particles at the membranes of infected cells, where they then pinch off and bud into round, enveloped particles. When they are in their immature stage, type C particles also resemble type A viruses, but after proteolytic maturation, which occurs during of after budding, a dense nucleocapsid core is formed. Type C particles also have surface projections, but they are barely visible, unlike those found on type B viruses.

Type D viruses resemble type B viruses in that they are formed intracellularly. However, the mature core is not centrally located in the virus, and surface projections are also less prominent.

Although lentiviruses possess a type C morphology, they are unique in that the viral nucleocapsid is bar-shaped or cone-shaped.

1.1.4 Classical Classification

Another method that is used to organize the Retroviridae is based upon the evolutionary relatedness of the viruses (51). The three subfamilies used in Classical Classification are the Oncovirinae, (further divided into 5 groups: the Avian leukosis-sarcoma group, Mammalian C-type group, B-type group, D-type

group, HTLV-BLV group), the Lentivirinae and the Spumavirinae. These seven divisions of retroviruses are made according to 1) virion structure, 2) whether the viral lifestyle is endogenous (passed onto offspring in germ line cells as an integrated provirus) or exogenous, 3) the type of cell receptor used for cell entry, and 4) the presence of oncogenes.

1.1.4.1 Oncoviruses

The Oncovirinae subfamily is divided into 5 groups of retroviruses, most of which cause tumor formation in their hosts. The division of avian leukosis-sarcoma viruses contains exogenous viruses of birds that have C-type morphology. Most of these viruses contain an oncogene in addition to gag, pol and env genes. Examples include Rous sarcoma virus (RSV) and Avian myeloblastosis virus (AMV).

Mammalian C-type viruses are both endogenous and exogenous, and have a wide range of mammalian hosts, including primates, rodents and also in some birds. However, no infectious human C-Type viruses have been identified to date. Specific grouping of C-Type viruses into the ecotropic or xenotropic groups is made according to the specificity of their receptors for different species. Ecotropic viruses are only capable of replicating in murine cells, whereas xenotropic viruses can replicate in most other species, except for mice. Furthermore, polytropic and amphotropic viruses use other classes of receptors not used by ecotropic or xenotropic species, and their species host range is broad, and includes mice. Mammalian C-type viruses contain only gag, pol and

env genes, with many isolates containing oncogenes. Examples of mammalian C-type viruses are Moloney murine leukemia virus (Mo-MLV) and Feline leukemia virus (FeLV).

The B-type virus group has only one infectious member: Mouse mammary tumor virus (MMTV). B-type viruses contain gag, pol and env genes, as well as an ORF of unknown function.

D-type viruses are exogenous viruses that cause immunodeficiencies in monkeys, and appear to only contain gag, pol and env genes. An example is Mason-Pfizer monkey virus (MPMV).

Members of the HTLV-BLV group (Human T-cell leukemia virus and Bovine leukemia virus) cause B-cell or T-cell lymphomas. In humans, HTLV is responsible for neurological disease. These viruses have an exogenous lifestyle. Besides containing gag, pol and env genes, at least two other proteins involved in gene expression are also encoded.

1.1.4.2 Lentiviruses

Lentiviruses are exogenous viruses that have no direct role in tumor generation. Diseases caused by lentiviruses include neurological and immune deficiencies, which generally progress slowly. Gene organization is complex in lentiviruses, for in addition to encoding the gag, pol and env genes, they also possess several other viral genes that help to regulate the life cycle. Examples of lentiviruses include Human immunodeficiency virus (HIV) and Simian immunodeficiency virus (SIV).

1.1.4.3 Spumaviruses

The Spumaviruses, or "foamy" viruses, are the least characterized of the retroviruses, and are called "foamy" because of the vacuolation that they cause in mammalian culture cells. Thus far, spumaviruses have not been found to be associated with any disease. Their genetic organization is complex, and includes genes coding for transactivating proteins.

1.2 General description of HIV-1

1.2.1 Discovery of HIV

HIV-1 was the first human lentivirus to be discovered. This virus, which is the etiologic agent of AIDS, was first described by two independent groups, one led by Luc Montagnier (189) from the Pasteur Institute in Paris, and the other led by Robert Gallo (100), from the NIH in Bethesda. The Paris group obtained a virus containing reverse transcriptase from the lymph node of a man with persistent lymphadenopathy syndrome, which they initially called LAV (Lymphadenopathy-associated virus). Some of LAV's characteristics included previously-reported characteristics of Human T-cell leukemia virus (HTLV). It is because of this that many investigators concluded that this new isolate, LAV, was really a form of HTLV. Additionally, in the same issue of the journal Science that reported the isolation of LAV, Gallo's group also reported the isolation of HTLV from patients with AIDS. Subsequently, it was discovered that Gallo's sample had been contaminated, and in 1984 his group reported the characterization of another human retrovirus from patients with AIDS. This virus, which they called

HTLV-III, was the same virus as LAV, and eventually LAV and HTLV-III were renamed HIV.

1.2.2 Worldwide prevalence

While the causative agent for AIDS was being identified and studied, the disease was spreading quickly though the human population. According to the World Health Organization/UNAIDS, at the end of 2001 there were 40 million people living with AIDS around the world. Sub Saharan Africa had the highest adult prevalence rate of AIDS, at 8.8% of the total population, or 28.1 million people. North America had an adult prevalence rate of 0.6%, or 0.92 million people.

1.2.3 Evolution of HIV

There are 2 types of HIV: HIV-1 and HIV-2 (50). HIV-1 is most prevalent in North America, Europe, Central Africa and Asia, while HIV-2 is mainly found in West Africa (47). HIV-1 and HIV-2 differ in their sequences by approximately 55%. In fact, HIV-2 has a higher sequence homology with Simian immunodeficiency virus (SIV), and antibodies from both SIV and HIV-2 cross-react (64), (142). It is believed that both HIV and SIV evolved from a common ancestor, with HIV-1 and HIV-2 entering the human population at separate points in time through cross-species transmission from primates to human (73).

The transmissibility and pathogenesis of HIV-2 is lower than for of HIV-1, and people infected with HIV-2 tend to live longer after they have developed

AIDS (184). In HIV-2-infected individuals, more neutralizing antibodies are found than in patients infected with HIV-1. This may be due to the major differences in envelope glycoproteins, which accounts for some of the difference in sequence homology between the two viruses.

There is also extensive genetic diversity of HIV-1. Far from being a single virus, HIV is actually a family of related virus types, or clades (also called subtypes) and strains. These subtypes (A through H) differ in about 30% of their total genetic sequence, and are not evenly distributed around the world but exist as distinct geographic clusters. Within subtypes, new strains are continuously being generated by HIV-1's very high rate of mutation and by genetic recombination, often viewed a serious problem in development of an AIDS vaccine.

Until recently, AIDS vaccine research has mostly focused on subtype B, found primarily in Europe, the Americas, Japan and Australia, while HIV-1 subtypes A, C, D and the recombinant subtype E are spreading rapidly in Africa and Asia and cause far more cases of AIDS globally.

1.2.4 Overview of genetic organization of HIV

1.2.4.1.The Long Terminal Repeat of HIV-1 proviral DNA

The Long Terminal Repeat (LTR) of proviral DNA is divided into 3 functionally distinct regions, which are known as U3 (-453 to +1), R (+1 to +98) and U5 (+99 to +185). In the U3 region, the viral transcriptional promoter elements consist of three domains: the core (TATA box and SP1 binding sites),

the enhancer (NF-κB binding sites) and modulatory regions such as AP-1 and NFAT-1 sites (174). Transcription starts from the R region, and both R and U5 regions are part of the 5' untranslated leader region.

1.2.4.2. Important elements of HIV-1 RNA

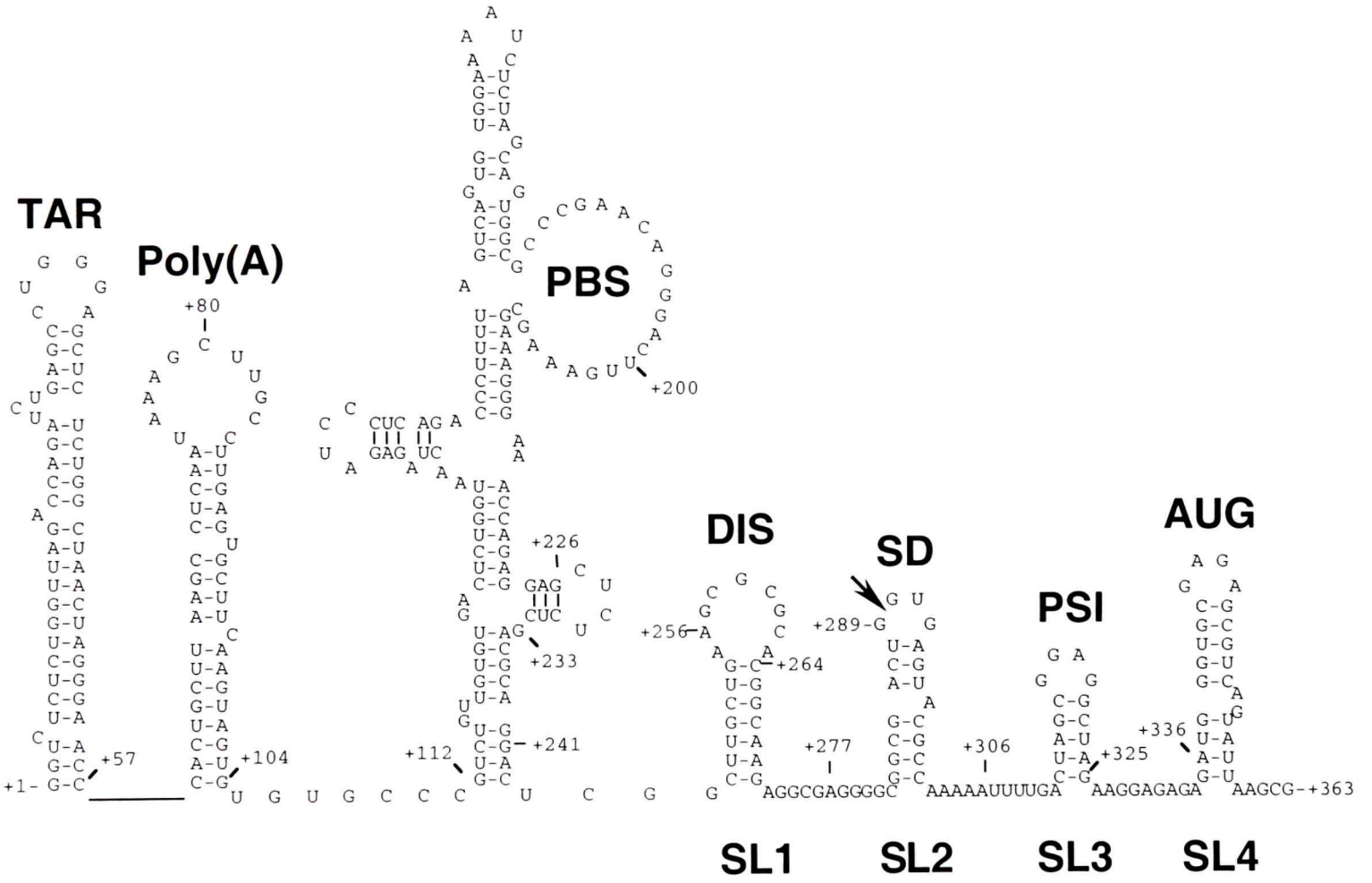
HIV genomic RNA is 9.2 kb in length. It has an m7G5'pppG$_m$p cap on its 5' end that is added by cellular transcription machinery during RNA synthesis (53). Internal methylation sometimes occurs on A residues, which may be important for splicing. Two identical copies of full length viral RNA are packaged into the virus.

The 5' R-U5 and Leader Region of HIV-1 RNA can be divided into a number of stem/loop structures that are important in transcriptional activation, viral RNA dimerization and encapsidation. These stem/loop structures are shown in Figure 1.3. The TAR stem-loop, found in R, primarily functions to regulate virus transcription through interaction with the viral Tat protein, whose binding promotes a high level of transcriptional activation (88). Also, the presence of the TAR stem structure may be an important factor in viral RNA packaging (116), as was shown when mutations were made in the lower portion of the TAR stem, resulting in a 10-25 fold lower encapsidation efficiency than in wild-type.

Downstream of TAR is the poly(A) hairpin, which may also be necessary for packaging of viral genomic RNA and/or stability of the viral RNA (67). Further downstream is the primer binding site (PBS), whose 18 nucleotides are perfectly complementary to the 3'-terminal nucleotides of tRNA^{Lys3}, and to which tRNA^{Lys3}

Figure 1.3. The 5' Leader sequence of HIV-1.

Adapted from (17).



Adapted from Berkhout, B. 1996. Prog. Nucleic Acid Res. Mol. Biol. 54: 1-34.

binds to prime reverse transcription. The PBS is part of an extended structure that may be involved in regulating the annealing of the primer tRNA, and in the initiation of reverse transcription, and this will be discussed in further detail in this thesis.

Downstream of the PBS region are four stem-loop structures (SL1, SL2, SL3 and SL4) that have both independent and overlapping functions. The SL1 stem-loop contains the palindromic DIS (dimerization initiation signal) hairpin sequence. DIS is critical for initiation of genome dimerization. Its dimerization function is thought to be initiated by a "kissing-loop" mechanism, thereby facilitating the interaction of sequences outside of the DIS region. NC may be involved in facilitating this hybridization, which involves two palindromic DIS sequences from two genomic RNA's initiating hybridization. (158), (190), (195). The major splice-donor site (SD) in SL2 is used to generate all sub-genomic spliced mRNAs (206), and as a result SL2 is present only in the full-length, unspliced viral genome. In SL3, the packaging signal Ψ plays an important role in the incorporation of genomic RNA into the virion (5), (163), through interaction with NC. SL4 contains the start codon (AUG) for Gag translation.

Important RNA elements are also found downstream of the 5' Leader region. Several downstream regions of the RNA contain splice acceptor sites, and at the 3' end of HIV-1 RNA, there is a polypurine tract (a run of at least nine A and G residues),followed by a polyadenylation site. Like the 5' capping group, the poly-A tail is added as a post-transcriptional modification by cellular machinery.

1.2.4.3. Organization of the viral genome

Figure 1.4 shows the genetic organization of HIV-1 DNA, viral genomic RNA, and the three open reading frames (ORF's) for viral structural proteins. The SL4 loop of the leader region (seen in Figure 1.3) contains the start codon for Gag, which codes for MA, CA, NC, p6, p2 and p1. The pol gene is located downstream of gag, in a different reading frame, and codes for viral catalytic proteins protease, reverse transcriptase and integrase. All gag and pol genes are transcribed into one unspliced message RNA, which is translated into the Gag precursor 90-95% of the time. The remaining 5-10% of the time, a minus one (-1) ribosomal frameshift event occurs in gag near the C-terminus of nucleocapsid, resulting in read-through and translation of the GagPol precursor.

Partially overlapping the pol gene is vif, which itself is overlapped by vpr. Next are portions of the genes coding for both tat and rev (the remaining parts are found within the env gene), and the gene coding for vpu. The last structural protein is env, which codes for the envelope glycoproteins gp120 (SU) and gp41 (TM), and it is located downstream of vpu. The accessory gene nef is found 3' to env, and overlapping into U3. An integration signal is found immediately downstream of U3, and this integration signal is an approximately inverted copy of the matching signal in U5.

Figure 1.4. HIV-1 proviral DNA, viral genomic RNA and viral structural proteins.

A. HIV-1 proviral DNA.

B. HIV-1 genomic RNA

C. HIV-1 structural proteins

Figure 1.4 A. Proviral DNA

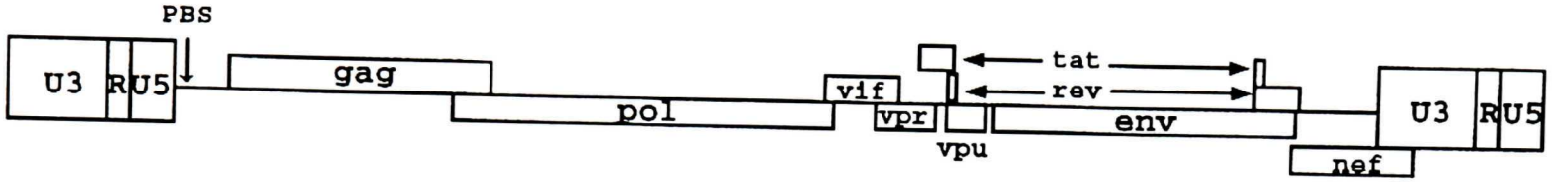


Figure 1.4 B. Viral Genomic RNA

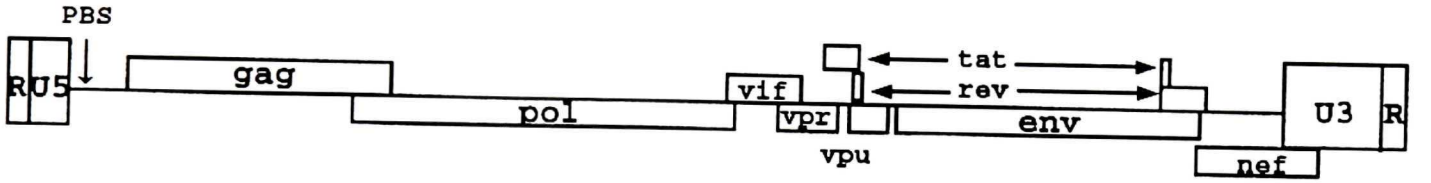
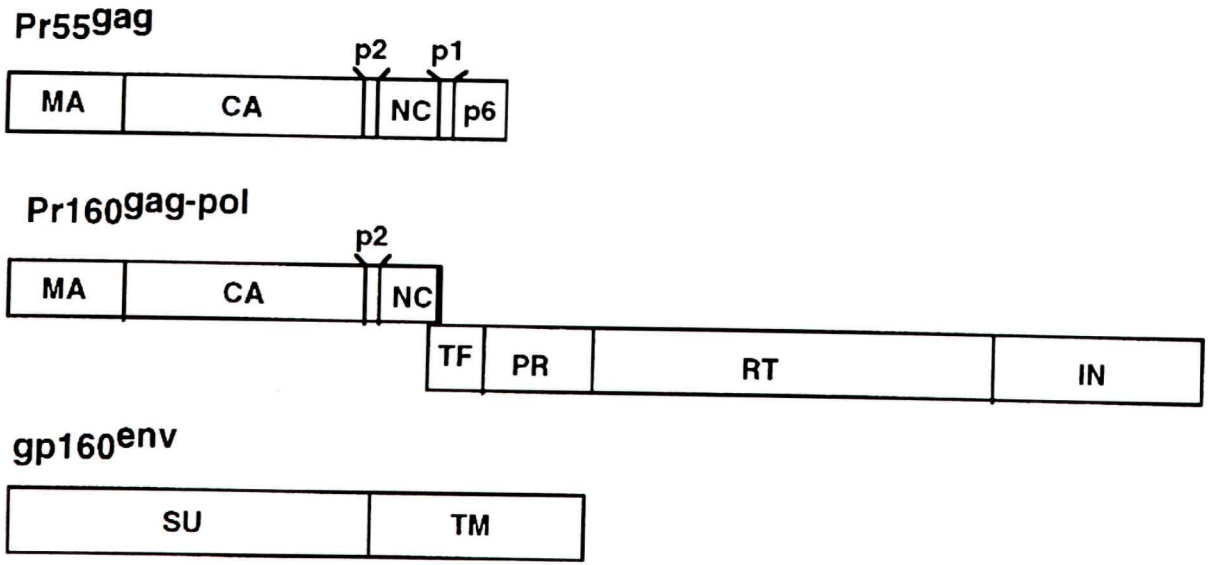


Figure 1.4. C. Viral Structural Proteins

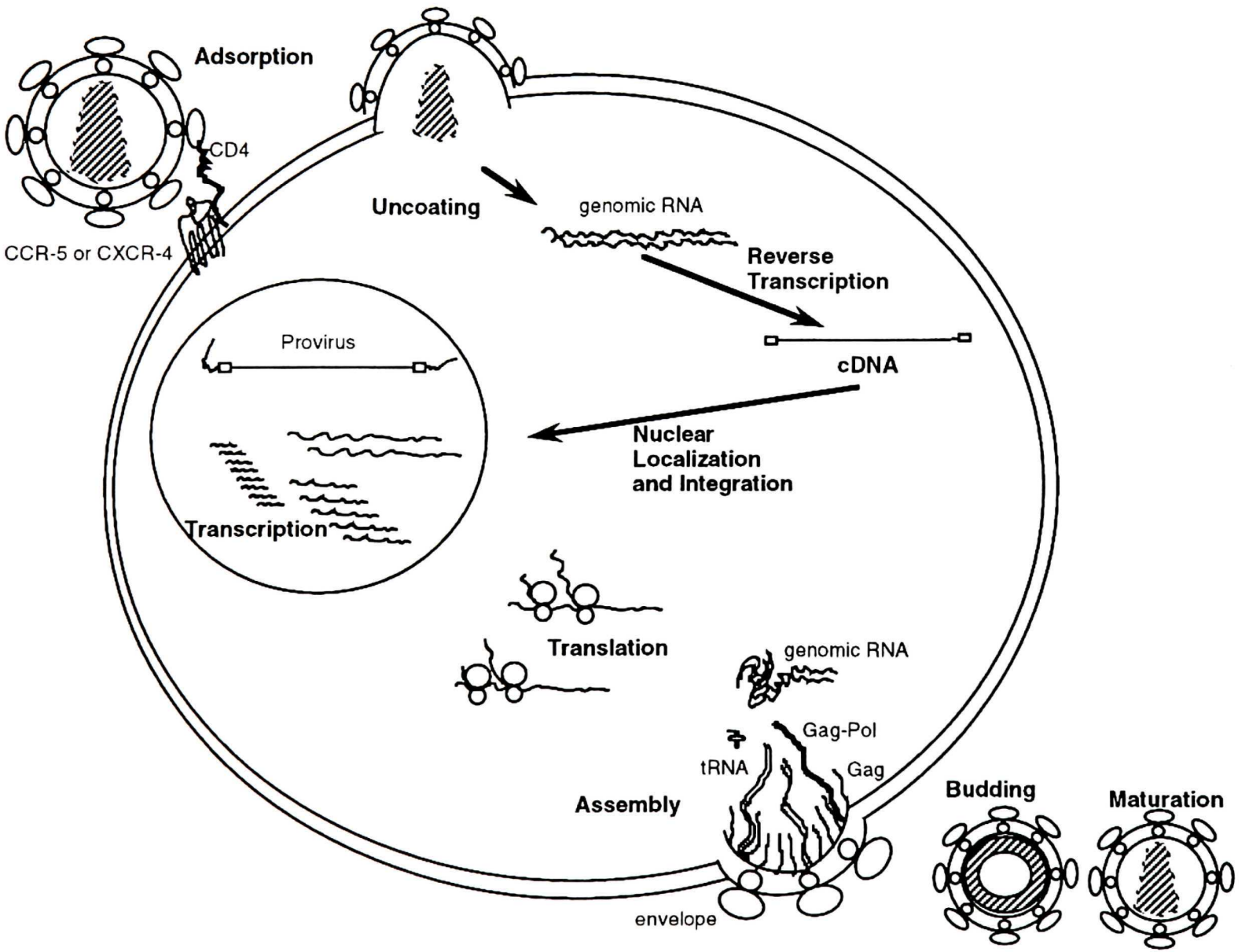


1.2.5 Overview of the HIV-1 life cycle

The life cycle of HIV-1 is summarized in Figure 1.5. Entry of HIV-1 into the target cell occurs following adsorption of the viral envelope protein gp120 to the cell surface receptor CD4. In addition to CD4, a specific chemokine receptor, most commonly CCR5 or CXCR4, must also interact with gp120. These interactions result in a conformational change in TM that promotes virus-cell membrane fusion, thereby allowing entry of the viral core (composed of two copies of viral RNA, $tRNA^{Lys3}$, and structural and enzymatic proteins) into the cytoplasm of the cell (40), (23) (275) (52), (242). Reverse transcription occurs in the cytoplasm, resulting in the conversion of viral RNA into double stranded proviral DNA. Reverse transcription is catalyzed by reverse transcriptase, which is primed by pre-packaged $tRNA^{Lys3}$. The eighteen 3'-terminal nucleotides of $tRNA^{Lys3}$ are complementary to the primer binding site (PBS), a region located at the 5' end of the viral RNA (208). Once the proviral DNA is synthesized, it is transported to the nucleus within a pre-integration complex composed of RNA and the viral proteins MA, IN, RT, and Vpr (96).

The pre-integration complex (particularly matrix and Vpr) is thought to provide the lentiviruses with the ability to infect non-dividing cells, since it actively transports proviral DNA into the nucleus (115), (30), (259). Conversely, other groups of retroviruses, such as the Oncovirinae, require nuclear-membrane breakdown (cell division) for transport of pro-viral DNA into the nucleus (169). Inside the nucleus, proviral DNA is integrated into the host's genome by integrase (27) near L1 and *Alu* elements (244), particularly near genes that are

Figure 1.5. The HIV-1 life cycle.


Adsorption
CD4
CCR-5 or CXCR-4
Uncoating
genomic RNA
Reverse
Transcription
cDNA
Provirus
Nuclear
Localization
and Integration
Transcription
Translation
genomic RNA
Gag-Pol
tRNA
Gag
Assembly
Budding
Maturation
envelope

activated in cells after infection by HIV-1 (227). Following integration, the provirus lies dormant until transcriptional activation occurs by viral regulatory proteins (88) and cellular transcription factors (219). Once activation takes place, cellular RNA polymerase II begins to transcribe viral mRNA from the viral promoter at the first nucleotide in the 5' R region. An early gene product Tat, binds to the TAR stem loop in the 5' LTR and greatly enhances the rate of transcription (88), (93), (138), which yields doubly-spliced RNA (1.8 kb), singly-spliced RNA (4.3 kb), and non-spliced RNA (9.2 kb). The early viral proteins Tat, Rev and Nef are produced from doubly-spliced RNA, and are exported out of the nucleus by cellular mechanisms. Singly-spliced RNA codes for the envelope proteins (gp120, gp41), and the auxiliary proteins Vpr, Vpu and Vif, while non-spliced RNA codes for the Gag and GagPol precursor proteins. Singly-spliced and non-spliced viral RNA's are transported from the nucleus to the cytoplasm by Rev, and in the absence of Rev, these transcripts cannot exit the nucleus (81), (87), (108), (180).

Translation of Env mRNA takes place in the endoplasmic reticulum, where gp160 is co-expressed and complexed with CD4. Cleavage of gp160 in the Golgi complex by a cellular protease, 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, $Pr55^{Gag}$ and $Pr160^{GagPol}$ are localized to the cell membrane, which is the site for viral assembly. Assembly involves the formation of Gag complexes, which incorporate GagPol, along with 2 copies of viral RNA, and $tRNA^{Lys}$. Budding takes place after assembly, and proteolytic processing of $Pr55^{Gag}$ and $Pr160^{GagPol}$, mediated by the viral protease, gives rise to a mature virus that is ready to infect the next cell.

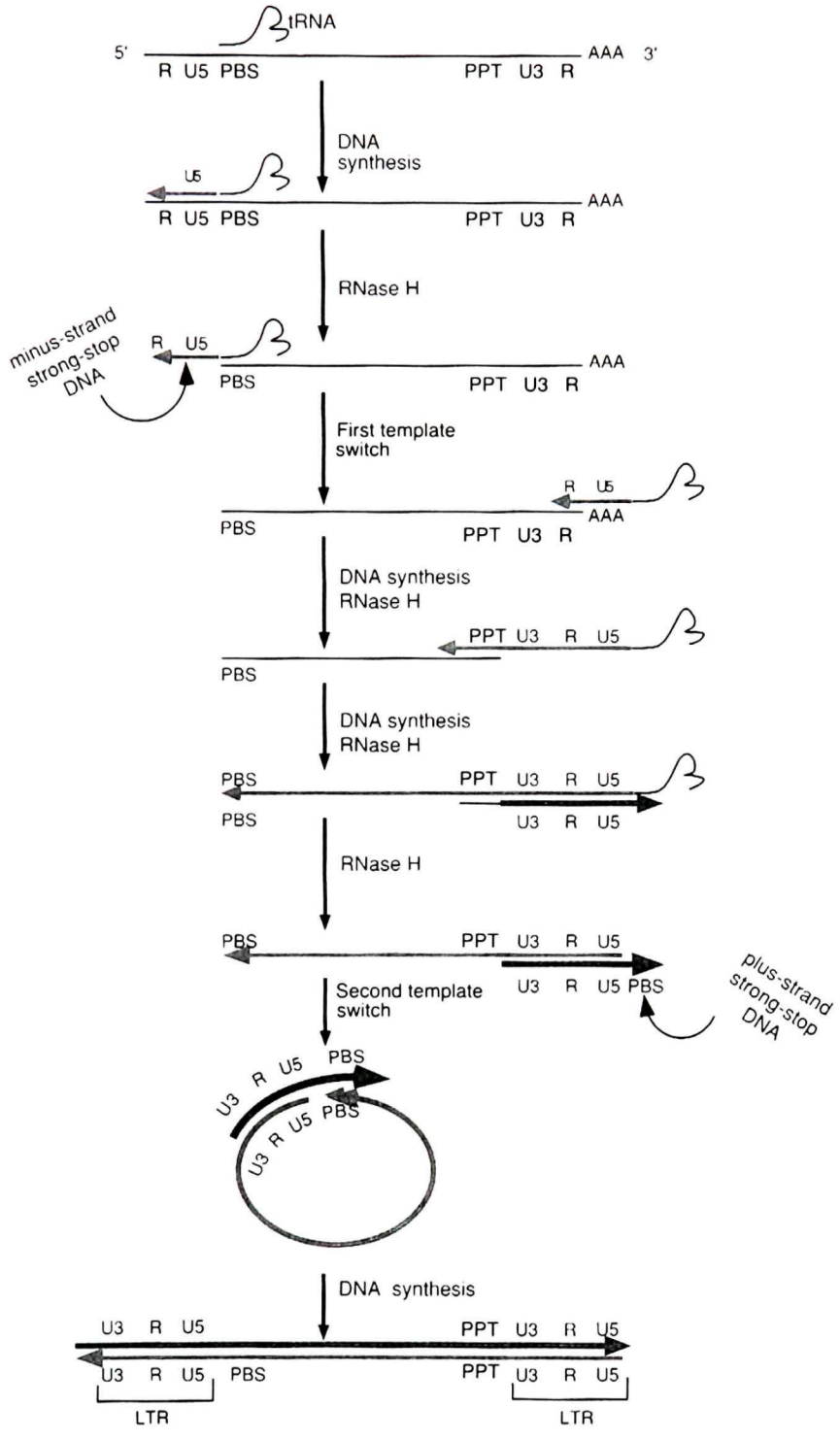
1.2.6 Reverse Transcription

Reverse transcriptase is both a RNA-dependent and DNA dependent DNA polymerase that catalyzes the synthesis of double stranded proviral DNA. Through its ribonuclease (RNase H) domain, this enzyme can also hydrolyze DNA-RNA hybrids. Figure 1.6 illustrates the different steps of reverse transcription as described below.

The primer for reverse transcriptase is $tRNA^{Lys3}$, whose 3' 18 terminal nucleotides are complementary to the primer binding site (PBS), located near the 5'end of the viral RNA (208). When $tRNA^{Lys3}$ is bound to the PBS and extended by reverse transcriptase, transcription of the 5' unique (U5) and repeat (R) regions occurs to produce minus (-) strong stop DNA (113), (268). The RNA template is digested by RNase H simultaneously with DNA extension, freeing the newly-synthesized DNA strand. The first template switch occurs, where the short nascent strand of DNA is translocated to the 3' end of the same RNA strand, or to another viral RNA strand. The first template switch is mediated by the R

sequence, which is identical at both ends of the RNA genome. This translocation event is also assisted by RNase H (24).

Figure 1.6. Reverse transcription in HIV-1.
Adapted from (54).


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
U3 R U5 PBS
plus-strand strong-stop DNA
Second template switch
U3 R U5 PBS
U3 R U5 PBS
DNA synthesis
U3 R U5
PPT U3 R U5
U3 R U5 PBS
PPT U3 R U5
LTR
LTR

Minus strand DNA synthesis continues through to the end of the PBS sequence on the viral RNA, still with simultaneous degradation by RNase H of the viral RNA template. There is an RNA region that remains associated with the newly synthesized minus strand DNA, known as the poly purine tract (PPT), which borders the U3 end. The PPT primes reverse transcriptase to synthesize plus strand DNA. Synthesis of plus strand DNA progresses to the end of the minus strand DNA template, and also through to the 18 nucleotides on the 3' end of the primer tRNA. This ensures that the new virus will have the same PBS as its predecessor.

Next is the second template switch, where the complementary PBS regions of both minus and plus strand DNA hybridize. The remainder of the plus strand of DNA is made, along with a small portion of the minus strand, and ultimately full length, double stranded proviral DNA is produced.

1.3. The Gag Proteins

1.3.1. Matrix (MAp17)

The matrix protein is cleaved from the N terminus of Gag by the viral protease. It has a molecular weight of 17 kDa, and is located between the capsid and envelope, lining the inner surface of the viral membrane. There are three known functions of the matrix protein: directing Gag to the cell membrane, transportation of the pre-integration complex to the nucleus, and anchoring of the Env transmembrane (TM) protein to the virion surface.

The ability of MA to target Gag and GagPol to the cell membrane appears to be due to a post-translational modification by the host cell enzyme N-myristyl transferase (29), (105), (197). This enzyme removes the methionine residue at the N-terminus of the Gag polyprotein shortly following translation, and covalently attaches myristic acid, a rare 14-carbon fatty acid, to the next glycine residue. Large deletions in the MA portion of Gag result in the re-direction of virus particles to the endoplasmic reticulum (85) or to the cytoplasm (238). However, replacement of the MA domain with myristic acid will restore viral assembly and targeting to the membrane (161) (263).

Basic amino acid residues located within the first 50 amino acids of MA which are involved in membrane localization, also function as a nuclear localization signal (NLS) (281), (259), (30). Post-translational phosphorylation of MA on serine and threonine residues results in the loss of its membrane-binding capacity because phosphorylation reveals a nuclear localization signal in the basic region that interacts with Rch1 (a cellular protein that facilitates rapid nuclear transport) (245). Phosphorylation of another MA residue, Tyr131, brings about an association with integrase, which is also a member of the pre-integration complex (99).

MA is also required for incorporation of Env into the virus (92), (182), most likely due to insertion of long cytoplasmic tails of transmembrane (TM) sub-units into the space between MA trimers (119). Replacing the first 2/3 of MA at the N-terminus with myristic acid does not affect processing or virion assembly and release, but it does inhibit Env incorporation and therefore renders the virus non-

infectious (276). Mutations at the carboxy-terminus of MA do not affect Env incorporation.

1.3.2. Capsid (CAp24)

The capsid protein is the major subunit of the viral core, with about 2000 molecules per virion. It is 24 kDa in size, and is released from central portion of $Pr55^{Gag}$ by the viral protease. Its role in the viral life cycle is a structural one, and studies in cell-free systems indicate that CA is capable of self-assembly because of its ability to form dimers and oligomeric complexes under proper pH conditions (31).

Within CA there is a highly conserved internal sequence of 20 amino acids called the major homology region (MHR), which plays an important role in particle assembly (273); it is highly conserved among retroviruses (200), (273). The MHR is essential for particle assembly, and may have a role in incorporation of GagPol precursors through interactions with Gag (240). It may also contribute to CA's membrane affinity through exposure of hydrophobic residues that contribute to the hydrophobic viral core (78).

The C-terminus of CA is important for dimerization, of Gag, possibly GagPol oligomerization, and viral assembly. Small deletions at the carboxy-end of CA result in a reduction of virus production, and mutations stretching into the MHR adversely affect the formation of the viral core (74).

N-terminal mutations do not prevent particle assembly, but do result in the production of virus with reduced infectivity, possibly due to the alteration of

morphology of the mature cone-shaped core when the p2 cleavage product at the C-terminus is deleted (22).

1.3.3. Nucleocapsid (NC)

Nucleocapsid is a nucleic acid-binding protein that has a molecular weight of 7 kDa. It is a product of the intermediary cleavage product p15, which is found at the carboxy-terminus of Gag. Other products of p15 include p6, p1 and p2. It is thought that the cleavage of p15 is the rate-limiting step in precursor processing (199).

There are many functions attributed to of NC. These include facilitating the annealing of primer $tRNA^{Lys3}$ onto the PBS, packaging of viral RNA (217), involvement in the tight packing of Gag molecules during viral assembly (118), facilitating template switching and reducing self-annealing of the minus (-) strand DNA template during reverse transcription (215), and possibly the facilitation of viral RNA dimerization (90), (91).

While the basic charge of NC gives it the ability to bind non-specifically to RNA, zinc fingers give NC its specific nucleic acid binding capacity (20). Both The NC protein HIV-1 and AMV NC proteins contain two zinc fingers of the CCHC type (193), while Mo-MuLV contains only one (117). 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 (48), (222). This signal is composed of three RNA hairpins located around the major splice donor site (19). 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 (70), (66), although its been suggested that both the flanking sequences and the zinc finger itself are required for dimerization in vitro (65).

There is specificity of NC proteins for their cognate RNA's. The specificity of HIV-1 NC for HIV-1 genomic RNA has been shown in vitro by replacing the MoMuLV NC sequence in MoMuLV proviral DNA with HIV-1 NC sequences. Co-transfection of this mutant MoMuLV with an untranslatable HIV-1 proviral DNA into mammalian cells produced mutant MoMuLV particles that packaged the HIV-1 genomic RNA. The reverse experiment, HIV-1 NC was replaced with MoMuLV NC produced similar results (21).

$tRNA^{Lys3}$ genomic placement also requires the presence of basic amino acid residues flanking the first Cys-His box (70), (157). NC likely unwinds the secondary structure of stem/loops in the PBS area of the genomic RNA making the PBS more accessible for $tRNA^{Lys3}$ (132). 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 transfer (106). Also, NC may play a role in forming the $tRNA^{Lys3}$ packaging binding site, possibly as part of a Gag/GagPol complex (125). The role of NC in packaging and placement of $tRNA^{Lys3}$ is discussed in further detail later in this thesis.

The tight-packing of Gag molecules that occurs during viral assembly is most likely mediated by NC. 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 (14).

1.3.4. NCp6

The p6 protein is a cleavage product of NC, making up the final 51 amino acids at the C-terminus of Gag. p6 assists in mediating efficient particle release (104) (154). The functions of the other cleavage products of NC – p2 and p1 – are still unknown.

1.4. The GagPol proteins

1.4.1.Protease (PRp10)

The viral protease is a 10 kDa aspartyl protease that processes Gag and GagPol into their mature forms. Experiments using site-directed mutagenesis to mutate the protease gene result in the production of non-infectious, immature virions containing only un-cleaved Gag and GagPol precursors (151) (172) (183).

Activation of protease requires dimerization. Once dimerized, protease is cleaved out of GagPol through an autocatalytic process (273), and the fully active dimer then targets other sites in the viral polyproteins. Eleven sites are recognized by protease – four in Gag and seven in GagPol. The regulation of polyprotein processing is due to both the amino acid sequence of the cutting site, and the three-dimensional conformation of the polyprotein. The rate of cleavage at different site varies. For instance, the first protein to be released from Gag is

NC, and this rate of cleavage (at the p2-NC junction) is approximately ten times higher than at other sites on the Gag precursor (199). Initial activity of protease is dependent upon the amount of GagPol and the rate of autoprocessing of protease, which may be influenced by adjacent p6 sequences (282).

Originally, it was believed that processing by the viral protease occurred only after the viral release. However, its been suggested that the efficient budding of HIV-1 virions require the initiation of proteolytic cleavage of viral precursors at the membrane of the infected cell prior to the release of the virion (143).

1.4.2. Reverse Transcriptase (RTp66/RTp51)

Reverse transcriptase is an RNA dependent DNA polymerase that catalyzes the synthesis DNA from single stranded RNA or DNA, and contains RNase H activity. It was first discovered in mammalian retroviruses in the 1970's, and has since been found to be encoded in the genomes of plant pararetroviruses, hepadnaviruses, and in retrotransposons (185) (178). The high degree of amino acid homology between different retroviral polymerases and RNase H sequences suggests that there is a high degree of structural and functional conservation (133).

Reverse transcriptase is processed in two cleavage steps from the GagPol polyprotein. First, p66 is cleaved, and forms a homodimer with another p66 subunit. A second cleavage event occurs near the C-terminus of one of the two p66 subunits, resulting in a heterodimer composed of one p66 sub-unit and

one p51 sub-unit (160). Although both homodimer and heterodimer forms display reverse transcriptase and RNase H activity, the p51 subunit alone is inactive and lacks the RNase H moiety of the protein (121) (122). In order for the catalytic activity of reverse transcriptase to begin, pre-packaged cellular $tRNA^{Lys3}$ (specifically packaged into the virus by reverse transcriptase sequences in GagPol) must be bound to the template at the PBS.

The structure of the p66 subunit, as determined by X-ray crystallography, resembles a right hand, with the polymerase domain subdivided into the fingers, palm, and thumb domains (134). The catalytic site for RT lies in a cleft in the p66 subunit that contains three aspartate residues (Asp-185, Asp 186 and Asp-110, brought together due to the conformation of the enzyme). This sequence is highly conserved among retroviruses and among other DNA polymerases. The connection domain in p66 holds the RT and RNase H domains together.

As reverse transcription is a crucial process in the life cycle of HIV-1, many drugs have been designed to interrupt the structure and function of this enzyme. However, development of drug resistance is a problem. Because of RT's lack of proof-reading mechanisms, heterogeneous virus populations are produced due to direct misincorporations of non-complementary bases, slippages of nucleotide strands, and frameshift mutations. This can result in the generation of mutant populations of virus that are unaffected by drugs. The rate of base misincorporation for HIV-1 RT is between 1 in 4000 to 1 in 7000 (13) (205) (214), whereas for E.coli DNA polymerase, which has proofreading capacity, the misincorporation rate is 1 in 100,000 nucleotides (12).

Two classes of reverse transcriptase inhibitors in clinical use are nucleoside analogs (such as AZT and ddI), which are believed to bind to the polymerase acitve site, and non-nucleoside inhibitors (such as nevirapine), which are non-competitive inhibitors of reverse transcriptase.

1.4.3. Integrase (INp32)

Integrase catalyzes the integration of double stranded proviral DNA into the host cell's genome. In vitro systems have shown that integrase is responsible for the cleavage of dinucleotides from the 3' ends of double stranded proviral DNA, for the creation of overhangs in host cell DNA, and for strand-transfer activity that joins proviral DNA and host cell DNA together. (82), (232).

In its active state, integrase is an oligomer, most likely a tetramer (210). It can be divided into 3 domains: the N-terminal domain (residues 1-55), the catalytic domain and the C-terminal domain. The N-terminal contains a Zn-binding site (2 cysteines and 2 histidines), and is highly conserved within the retrovirus family. Each integrase monomer contains a helix-turn helix structure that is similar to those found in other DNA-binding proteins. The catalytic domain contains a Asp-Asp-Glu motif which is also conserved among integrases, and is important for the cleaving and joining reactions of DNA that occur during the integration process (210). The C-terminal domain possesses non-specific DNA binding capabilities, and forms integrase dimers out of parallel monomers.

1.5. The Env proteins

Two viral proteins are encoded by the env gene: the outer surface glycoprotein (gp120) and the transmembrane glycoprotein (gp41). These two protein subunits are encoded by a single spliced mRNA of 4.3 kb. Translation of this mRNA yields a protein that is only 90 kDa, but becomes 160 kDa in size after co-translational glycosylation in the ER, followed by addition of oligosaccharide chains, and giving rise to the gp160 Env precursor protein (196) (231) (6). Cleavage of gp160 in the Golgi complex by a cellular protease produces the two mature glycoproteins, gp120 and gp41 (129).

1.5.1. gp120

The gp120 protein exists as a trimer or tetramer (77) (194) (226) (267) (154), with oligomerization occurring before the cleavage of the gp160 precursor protein. It appears that the oligomers remain noncovalently attached throughout transport to the membrane (79) (72) (255).

Viral entry into the cell is mediated by the binding of gp120 to specific receptors on the host cell surface. The major receptor is CD4 (63), (155) which is found on the surface of some T cell and some primary macrophages (174). Binding of gp120 to CD4, however, is not sufficient for viral entry; in addition to gp120-CD4 interaction, gp120 must also bind to cell surface chemokine co-receptors (seven transmembrane G-coupled proteins) (46) (213). The first co-receptor to be identified was CXCR4/fusin, which permits entry of T-tropic viruses (isolates that infect T cells but not macrophages) (89). Another co-receptor,

CCR5, is a major co-receptor for M-tropic viruses (isolates that infect macrophages but not T cells) (71). Other molecules that act as co-receptors for HIV-1 have also been identified (46).

Upon gp120/CD4 interaction, structural changes occur where the variable "V3 loop" (an important determinant of viral tropism) of gp120 becomes exposed. This helps co-receptor binding and in ensuing viral entry presumably by recognizing and interacting with the cognate co-receptor (46). V3 is probably not the only determinant of co-receptor specificity, since viral isolates with different V3 regions have been found to use the same co-receptors (41) (49) (192).

1.5.2. gp41

The gp41 protein functions in the fusion between the viral and cell membranes. Initiation of fusion is believed to be mediated by the fusion peptide, a hydrophobic, glycine-rich sequence of about 20 amino acids found at the N-terminus of gp41. Two hydrophobic repeat sequences within the fusion peptide domain are predicted to form coiled coils, as seen in most viral fusion proteins There are two 4-3 hydrophobic (heptad) repeat sequences within the gp41 ectodomain which are predicted to form coiled coils, as seen in most viral fusion proteins (37), and biophysical studies suggest that three N-terminal helices form an interior, parallel-coiled-coil trimer, while three C-terminal helices pack in the reverse direction into three hydrophobic grooves on the surface of this coiled coil (173), which was confirmed by crystallographic analysis (250), (269).This six-helix structure represents the fusion-active conformation of gp41 (97), (39).

1.6. Accessory proteins

Accessory proteins are proteins other than the replicative ones (Gag, Pro, Pol and Env) that are encoded by the retroviral genome. In HIV-1 the accessory proteins are vif, rev, tat, nef, vpu, and vpr. Their effects are either late in the viral life cycle or early in cell infection. Accessory proteins may function in specialized cell types, providing functions that are at least in part duplicative of a function provided by a cell protein. Sometimes the effect of accessory proteins is weak, and other times it is required to sustain viral replication. Accessory proteins can be divided into two groups based upon the timing of their expression. Tat, Rev and Nef are Rev-independent proteins, and are expressed early in the viral life cycle, while Vif, Vpr and Vpu are rev-dependent late proteins that are expressed during active viral assembly.

1.6.1. Rev-independent accessory proteins

1.6.1.1. Rev

Rev is the so-called "Regulator of viral protein expression". It is found mainly in the nucleus and nucleoli of the infected cell, but it is shuttled between the cytoplasm and the nucleus, and as such possesses a leucine-rich nuclear export signal (NES) (187), (87), (211), (257), (258). Its function is to promote the transport of unspliced viral mRNA from the nucleus to the cytoplasm, through interactions with the Rev Response Element (RRE), a complex step loop structure found in the Env gene (114), (181), (191), (278).

Why does HIV-1 require such a protein? The eukaryotic cell has evolved mechanisms to retain non-spliced mRNA's in the nucleus until splicing is complete, which is a problem for many retroviruses. HIV-1 proviral DNA contains only one Long Terminal Repeat (LTR) promoter element and it encodes only one genome-length primary transcript. The expression of Gag, GagPol, Env and certain accessory proteins requires the transport of unspliced or singly spliced viral RNA out of the nucleus to the cytoplasm for translation. Furthermore, unspliced viral RNA is also required for packaging into progeny virus. With Rev, this unspliced and singly spliced viral RNA can be brought out of the nucleus and into the cytoplasm (81), (179).

Tat, Rev and Nef are all products of completely spliced mRNA's which no longer posess the RRE and as such their transcripts are exported out of the nucleus by host cell proteins. The remaining ORF transcripts are transported to the cytoplasm by Rev, through a complex mechanism that requires the participation of many cellular and nuclear proteins. Yeast two-hybrid assays have shown a highly specific interaction between Rev's NLS and certain nucleoporins (94), (25), an interaction that is bridged by Crm1, a nuclear export protein that is important for Rev export (256).

1.6.1.2. Tat

The Tat transactivator protein is transcribed early in infection from 2 exons found in the env gene, and is independent of Rev function. It is 14 to 15 kDa in size and is required to increase steady state level of viral RNA transcription by

binding to the TAR RNA stemloop structure at the 5' end of the viral genome (218) (237), (236) (221). The loop itself is bound by a complex of cellular factors, and these interactions are most likely required to adopt a stable structure. Although the provirus also contains other regulatory elements recognized by cellular transcription factors (102), they are inefficient, and Tat increases RNA production by as much as 100-fold.

The interaction of Tat with viral RNA may increase the stability of the RNA polymerase to allow more efficient synthesis of full-length transcripts, or may increase the frequency of RNA initiation (112).

1.6.1.3. Nef

Nef is the last ORF at the 3' end of the HIV-1 genome. It is the first viral protein to be produced in the infected cell (149), and plays a critical role in viral infection (146). Nef is packaged into the virus at very low efficiencies, most likely non-specifically, although it has been suggested that Nef is involved in reverse transcription in an indirect way (2), (229). In the infected cell, Nef has several functions. It down-regulates cell surface CD4 (101), (228) which is believed to reduce CD4-Env complexes on the infected cell surface, facilitating the release of new HIV-1 virions (15) and preventing superinfection and cytopathy (1) (15). Nef also induces the down-regulation of cell surface MHC I receptors to inhibit the CTL-mediated lysis of the infected cell (159), and enhances viral infectivity in some cell types through a CD4-independent mechanism (239).

1.6.2. Rev-dependent accessory proteins

1.6.2.1. Vif

Vif is a 23 kDa protein that is produced from a singly spliced mRNA transcript that accumulates late in infection. The gene is located downstream of pol, and the protein accumulates late in infection (103). Mutation of Vif results in low viral titer and in the production of unstable replication intermediates, and also results in great reductions of viral DNA synthesis (55) (234), suggesting that Vif functions either before or during DNA synthesis. Interestingly, defects in the replication of Vif mutant viruses occur in some cell types (non-permissive) but not in others (permissive). This may be explained by the hypothesis that permissive cells provide a compensating factor for Vif-negative virus, or that Vif expression in non-permissive cells blocks an inhibitor of viral infectivity (55).

Vif may also play a role in viral assembly and/or maturation (120), a fact supported by Vif's association with the membranes of infected cells, and its interaction with the cytoskeleton and other viral components (144). Vif is not packaged in significant amounts, and appears to have no role inside the virus.

1.6.2.2. Vpr

Vpr is a 15 KDa virion-associated protein that is incorporated into viral particles through interactions with p6, and is present in numbers similar to those of Gag-related proteins (56), (201), (154). A major function of Vpr is the transport of the pre-integration complex to the nucleus (26). Vpr associates with the complex through interactions with the C-terminus of MA, and then localizes the

complex to nucleus via its nuclear localization signal (NLS) (42), (115). Vpr also displays the capability to disrupt the cell cycle in G2 phase, which may block the clonal expansion of anti-HIV-specific T cells (167), (168), (59) (216) (139).

1.6.2.3. Vpu

Vpu is made from the same singly spliced mRNA as the envelope glycoprotein (57), (230), (247), and is an integral membrane protein (55) (156). Posttranslational phosphorylation of Vpu causes rapid and selective degradation of CD4 for the release of Env subunits (271), (272), (55). Vpu also mediates the down regulation of MHC class I expression on the cell surface, which may protect the infected cell from recognition and killing by cytotoxic T lymphocytes (145). Virion release is also stimulated by Vpu and it has been proposed that the protein is an ion channel (156).

1.7.Primer tRNA's in Retroviruses

Cellular tRNA's are used in both retroviruses and retrotransposons as primers for reverse transcription. In retroviruses, the tRNA is selectively packaged into the virion, where it is placed onto the viral genome at the primer binding site (PBS), and is used to prime the synthesis of minus (-) strand proviral cDNA.

In both retroviruses and retrotransposons, the PBS is located at the 5' end of the RNA genome. The identities of the tRNA's used for reverse transcription have been, for the most part, determined by the sequence of the PBS. Table 1.1

lists some retroviruses and retrotransposons and their primer tRNA's. In avian sarcoma viruses (ASV) and avian leukosis viruses (ALV), $tRNA^{Trp}$ is the primer tRNA (265), (266) (203), (224). In HIV-1, the three $tRNA^{Lys}$ isoacceptors that are present in mammalian cells are packaged into the virus: $tRNA^{Lys1}$,$tRNA^{Lys2}$ and $tRNA^{Lys3}$. $tRNA^{Lys1}$ and $tRNA^{Lys2}$ differ by only one base in the anticodon stem, and are therefore grouped together as $tRNA^{Lys1,2}$. $tRNA^{Lys3}$ is the primer for reverse transcriptase in the lentiviruses, such as HIV-1, HIV-2, equine infectious anemia virus (EIAV), and simian immunodeficiency virus (SIV) (162), and in mouse mammary tumor virus (202), (264). Moloney murine leukemia virus (Mo-MuLV) uses $tRNA^{Pro}$ as primer for reverse transcription (109), (251), but isolates that use primers other than $tRNA^{Pro}$ have been found. For example, $tRNA^{Gln1}$ has been identified as an alternate primer for a Mo-MuLV isolate that shows wild type replication kinetics (58), and endogenous murine retroviruses that possess PBS's complementary to $tRNA^{Lys3}$, $tRNA^{Phe}$ and $tRNA^{Gly}$ have also been reported (58). Thus, it appears that the specificity for cognate tRNA's is lower in mouse retroviruses than in HIV-1 and in the avian sarcoma viruses.

"Select packaging" refers to the increase in the concentration of a particular tRNA to the total tRNA population, going from the cytoplasm to the virus. For example, the relative concentration of $tRNA^{Lys}$ in the cytoplasm of a mammalian cell is 5-6% of total tRNA, whereas in HIV-1, 50-60% of viral tRNA is $tRNA^{Lys}$ (176). In AMV, primer $tRNA^{Trp}$ makes up about 32% of total viral tRNA, while in the cytoplasm, only 1.4% of tRNA is $tRNA^{Trp}$ (265). In AKR murine leukemia virus (AKR-MuLV), the primer tRNA is enriched to a lesser degree,

Table 2.1. Retroviruses and Their Primer tRNA's

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

where 5-6% of cytoplasmic tRNA is $tRNA^{Pro}$, and in the virus the concentration is only 12-24% (265). 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 (126). In HIV-1, estimates of 20 molecules of $tRNA^{Lys}$/virion have been reported (126), but these numbers should remain estimates because the viral population studied may not be homogeneous for packaging of either genomic RNA or $tRNA^{Lys}$. Up until recently, there were no known functions of $tRNA^{Lys1,2}$ in the HIV-1 life cycle. However, recent evidence (reported in chapter 3 of this thesis) shows that $tRNA^{Lys1,2}$, like $tRNA^{Lys3}$, has a stabilizing effect upon GagPol when the tRNA's are overexpressed in HIV-1 produced from COS7 cells.

1.7.1.Factors involved in the packaging of $tRNA^{Lys3}$ in HIV-1

1.7.1.2. HIV-1 genomic RNA

There is no evidence to support a role of genomic RNA in the packaging of $tRNA^{Lys3}$ in HIV-1. It has been shown that primer tRNA packaging occurs in the absence of genomic RNA incorporation in ASV (204), MuLV (166) and in HIV-1 (136), (177). However, it is not known if the actual $tRNA^{Lys3}$ molecules used for reverse transcription are placed onto the PBS before or after packaging.

1.7.1.3. Role of reverse transcriptase sequences in $tRNA^{Lys3}$ packaging

The RT sequence within GagPol is an obvious candidate for involvement in $tRNA^{Lys3}$ packaging into HIV-1 because of the interaction of mature RT with

primer $tRNA^{Lys3}$ during reverse transcription. Both *in vitro* and *in vivo* work indicate the existence of such a role.

The *in vitro* studies deal with interactions between purified components, ie. mature RTp66/p51 and $tRNA^{Lys3}$. Both the p66 and p51 peptides in mature RT are divided into structural domains based upon x-ray crystallography. The RT peptides resemble a left hand, which is reflected in the names of the subdomains, termed the finger, palm, thumb, connection and RNaseH domains (134), (153). The RNaseH domain is not present in the p51 subunit. The determination of which of these regions within RT specifically interact *in vivo* with either the anticodon loop or the 3' terminus of $tRNA^{Lys3}$ have been investigated using in vitro studies.

Cross-linking (188), (76), (188) and enzymatic probe studies (10), (11), (223), (274) have shown that interactions exist between mature reverse transcriptase and the anticodon, TΨC and D loops, and 3' end of free $tRNA^{Lys3}$

In vitro studies have also indicated that both the p66 and p51 subunits of mature HIV-1 RT can interact with $tRNA^{Lys3}$ (274), (212), (223), (188), (75), (11), although the p66 subunit appears to bind to $tRNA^{Lys3}$ with a higher affinity than the p51 subunit (277).

Another group found evidence for an *in vitro* interaction between a small crevice of the thumb domain of p66 and the anticodon loop of $tRNA^{Lys3}$ (7), and mutations made in this crevice at K249 and R307 inhibited the interaction. However, the role of the $tRNA^{Lys3}$ anticodon in these interactions is uncertain since these same mutations had no effect upon $tRNA^{Lys3}$ packaging *in vivo* (148).

The *in vitro* studies described indicate an important interaction between the RT thumb subdomain and either the anticodon or the 3' terminus of $tRNA^{Lys3}$. *In vivo* studies have also indicated the importance of RT sequences in primer tRNA packaging. Selective packaging of primer tRNA does not occur in RT-negative ASV (203) or RSV (225), in a mutant MoMuLV which lacks 40% of the RT sequence from the C-terminus (164), or in an RT-negative HIV-1 (176).

Since the presence of RT is required for primer tRNA packaging, and since viral assembly occurs with larger precursor molecules which are not cleaved until budding, the GagPol precursor is more likely to be involved in this process since it contains the RT sequences. Studies on $tRNA^{Lys3}$ packaging have supported this hypothesis, and this was first shown when HIV particles composed of only Gag did not selectively package tRNALys, while particles composed of both Gag and GagPol did (176). Also, inhibition of HIV-1 protease or of genomic RNA packaging did not prevent the select viral packaging of primer $tRNA^{Lys3}$ (176). C-terminal deletions of GagPol which included the integrase domain and the RNaseH, connection domains of RT did not affect $tRNA^{Lys3}$ packaging, but further deletion of the RT thumb subdomain in GagPol severely inhibited $tRNA^{Lys3}$ incoproration into the virion (148). Furthermore, an earlier *in vivo* study also showed that mutations in both the thumb and connection domains resulted in a decrease of $tRNA^{Lys3}$ packaging (177), although in this case, GagPol packaging was also abolished.

1.7.1.4. Nucleocapsid sequence

Nucleocapsid sequences are involved in a number of retroviral processes, such as genomic RNA packaging and dimerization, strand transfer of minus strong stop cDNA and primer tRNA annealing (66), (106). There is also evidence suggesting that the NC sequences in Gag may contribute to a $tRNA^{Lys3}$-binding site associated with packaging (125). An HIV-1 mutant was constructed whereby the two Cys-His boxes and the linker region of NC were deleted. Transfection of this proviral DNA into COS7 cells produced virus that packages GagPol, but not $tRNA^{Lys3}$. Packaging of $tRNA^{Lys}$ was rescued by co-transfecting with wild-type Gag. This may indicate a role of NC in the Gag precursor in forming a $tRNA^{Lys}$-binding site, possibly as a part of a GagPol-Gag complex. However, because of the size of the deletion (DK14-T50), protein configuration is probably altered, and this may also produce Gag/GagPol particles that are unable to retain $tRNA^{Lys}$.

1.7.1.5. Lysyl-tRNA synthetase

In the mammalian cell, tRNA's are not found in a free state, but are bound to components of the translational machinery, and are channeled from one component to another (241). These components may involve ribosomes, elongation factors and aminoacyl-tRNA synthetases. Elongation factor 1-α is packaged into HIV-1 through interactions with Gag (43). However, it is unclear how this protein would be involved in selectively packaging $tRNA^{Lys}$ into the virion, since it binds to all aminoacylated tRNA's. Lysyl-tRNA synthetase, which is the enzyme that aminoacylates $tRNA^{Lys}$, has also been shown to be

specifically packaged into HIV-1 through interactions with Gag (35), and therefore it may be the signal that targets $tRNA^{Lys}$ isoacceptors for incorporation into HIV-1.

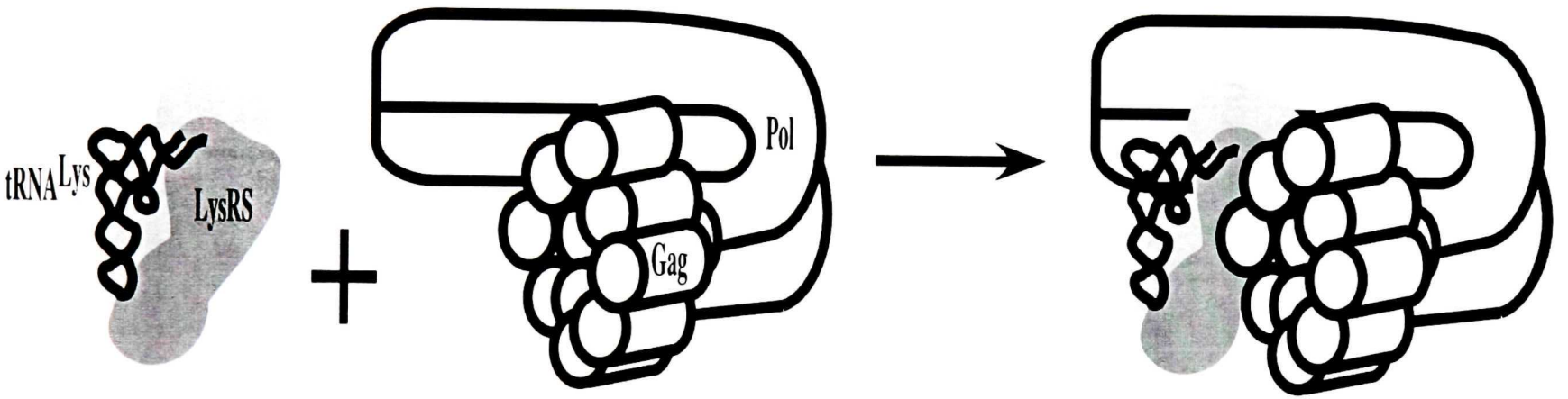
Gag alone is sufficient for packaging LysRS into Gag particles (35), but the additional presence of GagPol is required for $tRNA^{Lys}$ incorporation (176). It is possible that GagPol stabilizes the Gag/LysRS/$tRNA^{Lys}$ complex, since GagPol interacts with both Gag (198), (235) and $tRNA^{Lys}$ (176), (148). A model for the complex that may occur during $tRNA^{Lys}$ packaging is shown in Figure 1.7. The figure assumes that both Gag and GagPol multimerize, which is known to occur when facilitated by RNA, during viral assembly (248). The figure also shows LysRS dimerization, which has been shown to occur *in vivo* (45), (44).

While the major cytoplasmic LysRS has an apparent molecular weight of 68,000 the viral LysRS has an apparent molecular weight of 63,000, and since this truncation occurs in the absence of viral protease, a cellular protease may be involved (35). Truncation of LysRS is not a factor in $tRNA^{Lys}$ packaging since inhibition of LysRS truncation does not affect $tRNA^{Lys}$ incorporation into HIV-1 (36).

A limiting factor for the amount of $tRNA^{Lys}$ packaged into HIV-1 appears to be LysRS. The virion contains approximately 20 molecules of $tRNA^{Lys}$, averaging 12 molecules of $tRNA^{Lys1,2}$ and 8 molecules of $tRNA^{Lys3}$ (126). When an exogenous $tRNA^{Lys3}$ gene is expressed with HIV-1 proviral DNA in transfected cells, the number of $tRNA^{Lys3}$ molecules/virion can be doubled, but the number of $tRNA^{Lys1,2}$ molecules/virion decreases accordingly, maintaining the same number

1.7. Model of the $tRNA^{Lys}$/LysRS/Gag/GagPol packaging complex.

Based upon findings described in the text, this figure is a postulated model for the $tRNA^{Lys3}$ packaging complex. The model consists of a Gag/GagPol complex interacting with a $tRNA^{Lys}$/LysRS complex. This figure hypothesizes that Gag interacts with LysRS, and GagPol interacts with $tRNA^{Lys}$, and that the complex is carried into the assembling virion.


tRNALys
LysRS
Pol
Gag

of total $tRNA^{Lys}$/virion (126). However, when cytoplasmic LysRS is increased by transfecting cells with an exogenous LysRS plasmid plus HIV-1 proviral DNA, LysRS concentration in the virus increases, and viral $tRNA^{Lys}$ doubles, with packaging of all $tRNA^{Lys}$ isoacceptors increasing (98).

The ability of $tRNA^{Lys3}$ to be aminoacylated is directly correlated with its ability to be packaged into HIV-1. Mutations in the $tRNA^{Lys3}$ anticodon have been shown to strongly inhibit charging of $tRNA^{Lys3}$ by LysRS in vitro (249), (243) and in vivo (127), (135), with the order of decreasing in vivo aminoacylation and packaging ability being as follows: wild type SUU (100%) >SGU (49%) >CGU (40%) > SGA (0%) and CGA (0%) (135). This supports the conclusion that the productive interaction of $tRNA^{Lys3}$ and LysRS is important for $tRNA^{Lys3}$ incorporation into HIV-1.

1.7.2. The annealing of primer $tRNA^{Lys3}$ to genomic RNA

1.7.2.1. The anticodon loop and deacylation of $tRNA^{Lys3}$

The anticodon of $tRNA^{Lys3}$ may act as a signal for genomic placement. Both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ are packaged with the same efficiency (126), regardless of their different anticodons (SUU and CUU, respectively, where S=mcm^5S^2U). However, only $tRNA^{Lys3}$ is used as the primer for reverse transcription. Mutations made in the $tRNA^{Lys3}$ anticodon, where SUU was changed to CUA, prevented the mutant tRNA from acting as a primer in vivo (127), but it did not affect its packaging (126), (127).

In order for $tRNA^{Lys3}$ to act as a primer, it must be deacylated. In fact, all detectable $tRNA^{Lys}$ in HIV-1 is deacylated (126), while all detectable $tRNA^{Lys}$ in an infected cell is acylated (126). It is unknown whether the deacylation of $tRNA^{Lys3}$ occurs inside the virus, or in the cell, prior to packaging.

1.7.2.2. Reverse transcriptase

RT sequences may be involved in the genomic placement of $tRNA^{Lys3}$, since mature RT interacts with the primer during the initiation of reverse transcription. Several *in vitro* studies have indicated that interactions exist between mature RT and the anticodon of purified $tRNA^{Lys3}$ (11), (10), (223), (274). Also, the acceptor stem of $tRNA^{Lys3}$ (which contains the sequence that is complementary to the PBS) is digested by RNase A in the presence of retroviral RT, suggesting a partial destabilization of this region by the RT (223). This destabilization may be important *in vivo* in facilitating genomic placement of $tRNA^{Lys3}$. Interactions between RT and the primer's anticodon may also play a role in tRNA destabilization prior to genomic placement (223).

In vivo studies in ASV and HIV-1 have shown that the absence of RT abolishes genomic placement of primer tRNA (176), (68). However, the absence of RT also results in the loss of select packaging, and this thesis will show that a decrease in $tRNA^{Lys3}$ concentration in the virus results in the reduction of genomic placement. Thus, any involvement of RT in the genomic annealing of $tRNA^{Lys3}$ in HIV-1 cannot yet be ruled out. Although the absence of RT protein in HIV-1 and ASV results in the reduction of genomic placement, this is not the

case in Mo-MuLV. In Mo-MuLV, RT mutants lose the ability to selectively package primer $tRNA^{Pro}$, but genomic placement of tRNA is unaffected (164), (165). Therefore, RT may not be involved in primer placement in Mo-MuLV.

If RT does in fact play a role in facilitating the genomic annealing of primer tRNA in human and avian retroviruses, it likely does so as part of the GagPol precursor protein, since genomic placement occurs in protease negative virions (60), (128), (246).

1.7.2.3. Nucleocapsid

Extensive denaturation of both the $tRNA^{Lys3}$ and the U5-PBS-leader stem loop likely occurs during the annealing of $tRNA^{Lys3}$ to the viral RNA genome. *In vitro* studies have shown that NC facilitates the annealing of $tRNA^{Lys3}$ to genomic RNA (70), most likely by unwinding the secondary structure of stem/loops in the PBS area of the genomic RNA (132), and not by unwinding the secondary structure of tRNA (NC has only a subtle effect upon destabilization of tRNA alone) (147), (38), (254), (111). NC-aided genomic annealing of $tRNA^{Lys3}$ occurs in a concentration-dependent manner *in vitro* (171), and it appears that the NC-$tRNA^{Lys3}$ interaction is non-specific, ie. the interaction results from the ability of NC to interact with any RNA (186).

Evidence for a role of NC in primer placement has been found for other retroviruses, including RSV (primer $tRNA^{Trp}$) and Mo-MuLV (primer $tRNA^{Pro}$) (204). For both Mo-MuLV (69) and HIV-1 (70), *in vitro* annealing of primer tRNA is independent of the presence of Cys-His boxes of NC, which are important for

genomic RNA packaging (18) and the annealing step during minus-strand transfer (107), but is dependent upon basic amino acids flanking the first Cys-His box (or the only Cys-His box in the case of Mo-MuLV). This observation that has been confirmed *in vivo* in HIV-1 (32). It has also been shown that inhibition of $tRNA^{Lys3}$ annealing was affected to a much greater extent when mutations were made in the basic amino acids flanking the first Cys-His box in Gag nucleocapsid sequences compared to GagPol nucleocapsid sequences (32). In fact, it has been shown that Gag alone is sufficient for annealing $tRNA^{Lys3}$ to viral RNA in vitro (90).

Although the initial *in vivo* annealing of $tRNA^{Lys3}$ to the PBS occurs in the absence of precursor proteolysis (125), subsequent exposure of the annealed $tRNA^{Lys3}$ to mature NCp7 is required for optimal placement, ie, for the most efficient initiation of reverse transcription (34).

1.7.2.4.Lysyl-tRNA synthetase

As mentioned above, LysRS is packaged into HIV-1 along with $tRNA^{Lys}$. However, viral LysRS (63,000Da) is smaller than the cytoplasmic form (68,000 Da), and the truncation of viral LysRS is not due to the viral protease (35). The purpose of truncation is unknown, but regarding $tRNA^{Lys3}$ genomic placement, there are two possibilities. First, truncation may be required for the initial annealing of $tRNA^{Lys3}$ to the PBS, where the cleavage of LysRS may be required for the release of $tRNA^{Lys3}$ from the packaging complex. Alternatively, LysRS itself may play a role in annealing.

The second possibility is that the truncated form of LysRS is required for the optimization of initiation of reverse transcription by deacylating the packaged $tRNA^{Lys3}$, which is completely acylated in the cytoplasm and completely deacylated in the virus (126). Deacylation is required to free the 3' terminus of $tRNA^{Lys3}$ so that is can be extended by reverse transcription.

1.7.3. Interaction between viral genomic RNA and $tRNA^{Lys3}$ for placement

In retroviruses, packaged primer tRNA is found both in a free state or bound to the viral genome (203). Although it has been demonstrated that primer tRNA is selectively packaged in the absence of genomic RNA packaging in both HIV-1 (171) and MuLV (166), it is not known whether the actual placement of the two tRNA molecules used to prime RT are annealed to the PBS's before or after viral packaging.

The sequence of the HIV-1 PBS is complementary to the eighteen nucleotides on the 3' end of $tRNA^{Lys3}$. Mutational analysis has shown that the first nine nucleotides at the 5' region of the PBS sequence are essential for the initiation of reverse transcription in HIV-1 (209), shown when mutant HIV-1 proviral DNA lacking the first 9 nucleotides of the PBS was transfected into COS cells yielded in the production of noninfectious virus. Further studies have suggested that the first 6 of these nucleotides are sufficient for the production of infectious viral particles (261) However, these viral particles replicate with delayed kinetics, and eventually revert back to wild-type PBS sequences.

The sequence of the PBS alone is not sufficient to determine which primer tRNA will be used. Studies conducted with HIV-1 (68), (170), (262) and ALV (270) have shown that changing the entire 18 nucleotide sequence of the PBS to be complementary to a tRNA other than the natural primer produces viruses that are infectious, but with slow initial replication kinetics. During prolonged culture of these PBS mutants, eventual PBS reversion occurs, and the sequence is changed back to being complementary to the more favored wild type primer tRNA, restoring wild-type replication rates (68), (170), (262), (270). The reversion step most likely occurs from the initially rare use of the natural primer tRNA during plus-strand synthesis of strong stop DNA. The model of reverse transcription presented earlier in this thesis (Figure 1.6) predicts that the 18 terminal 3' end nucleotides of $tRNA^{Lys3}$ determines the sequence of the newly generated PBS.

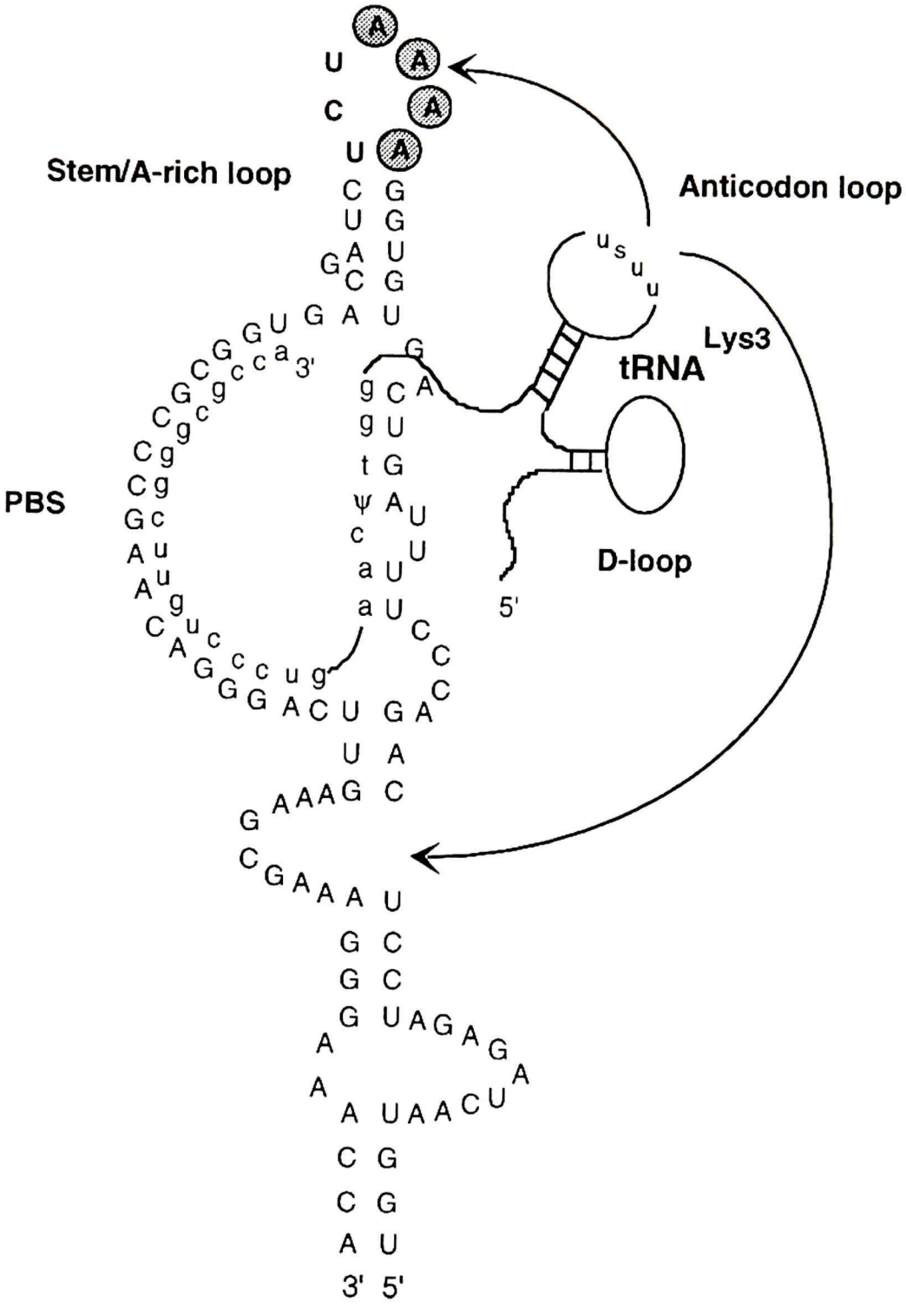
The preference of natural primer tRNA over others is seen even when the alternate tRNA is packaged in amounts similar to the wild-type primer tRNA. In HIV-1, $tRNA^{Lys1,2}$ is the most abundant tRNA in the virus, and yet it is not used as the primer for reverse transcription, even though the first 8 nucleotides of the PBS are complementary to $tRNA^{Lys1,2}$, meeting the minimum sequence complementarity requirements between its acceptor stem and the PBS (261). Furthermore, changing the PBS so that it is complementary to $tRNA^{Lys1,2}$ does not yield virus that stably uses $tRNA^{Lys1,2}$ as a primer (170), (68), (262).

Some MuLV systems may not have the same degree of stringency regarding primer tRNA utilization and PBS reversion as seen in HIV-1 and ALV.

For example, it was shown that a replication-defective retroviral vector derived from MuLV can use various cellular tRNA's as primers, while replicating with equal efficiencies (175). The vector contained only the LTRs, PBS, and some leader sequence of MuLV, as well as a neomycin gene, with viral proteins provided by an RNA packaging line. The resulting particles could only undergo one round of infection, with the production of G418-resistant cells used as evidence of reverse transcription. Vectors with PBS's complementary to tRNA's other than the natural primer $tRNA^{Pro}$ could produce as many G418-resistant cells as one with the natural PBS. The MuLV-based retroviral vector's greater ability to use alternate primer tRNA's could reflect the previously discussed finding that the restriction on the type of primer tRNA used in murine leukemia virus may not be as inflexible as for HIV or avian retroviruses.

The complementarity of the genomic RNA PBS sequence and tRNA acceptor stem sequence are not the only factors that determine which tRNA will be used to prime reverse transcription; other interactions between $tRNA^{Lys3}$ and genomic sequences have been found to be important. These interactions are shown in Figure 1.8 (adapted from (178)). They include that of the USUU of the anticodon loop of $tRNA^{Lys3}$ which interacts with an A-rich loop found in downstream of the PBS (152), and also with another much-studied A-rich region in the U5 stem loop upstream of the PBS, which was determined using chemical and enzymatic probes, and computer modeling (130), (132). Also shown in this figure is the proposed interaction between the TΨC loop of tRNA and a U5 region upstream of the PBS, first proposed based upon interactions seen in studies with

Figure 1.8. Interactions between $tRNA^{Lys3}$ and HIV-1 genomic RNA.


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

$tRNA^{Trp}$ and the genomes of ALV and ASV (3), (4). Although this model is widely accepted, criticisms have been raised stating that the numerous interactions between discontinuous regions of viral RNA and continuous regions of $tRNA^{Lys3}$ would result in a knot from the multiple RNA/tRNA interactions. This would make DNA elongation from $tRNA^{Lys3}$ by reverse transcriptase difficult without breaking and unwinding the tRNA with a topoisomerase, whose existence has not been shown (80).

In any case, the U5 A-rich loop does appear to play a role in determining which tRNA can be used as primer in HIV-1. Casey Morrow's lab has shown that infecting cells with a mutant virus that has been altered at both the PBS and the A-rich loop to be complementary to $tRNA^{His}$ at the acceptor stem and anticodon loop, respectively, results in the production of infectious virus that stably maintains a $tRNA^{His}$ PBS, without select packaging of $tRNA^{His}$ (260). In addition to the A-rich loop and PBS being altered in this case, some additional spontaneous mutations in the regions surrounding the PBS occurred during replication, which seemed to increase the rate of replication from initially low to almost matching that of wild-type. While this approach did not work for a number of other tRNA's tested, it also worked for $tRNA^{Meti}$ (141).

One interpretation of these U5 A-rich loop studies is that the compensatory mutations occuring during prolonged culturing result in the production of virus whose altered genomic RNA is less stable than that of the wild-type primer/PBS RNA (16). Therefore, placement of the new primer tRNA species onto the altered PBS could be more efficient than in the wild-type virus.

This would explain why selective packaging of these alternate primer tRNA's is not required, whereas in a wild-type virus, select packaging of $tRNA^{Lys3}$ is directly correlated with the virus's ability to primer reverse transcription (98). This could also clarify why some primer tRNA's are stably used in these experimental systems, and why some cannot be utilized, ie. the new structures and free energies resulting from both experimental and compensatory mutations may not destabilize the PBS region enough, and a higher viral concentration of the new primer tRNA may be required for efficient placement.

Although there appears to be an important role for the U5 A-rich loop in allowing tRNA's other than $tRNA^{Lys3}$ to be stably used as a primer for reverse transcription in HIV-1, there are several reasons to believe that the stem/loop and structure rather than sequences are involved. First, although all lentiviruses examined (HIV-1, HIV-2, SIV, FIV, EIAV) use $tRNA^{Lys3}$ as a primer, only HIV-1 contains the A-rich loop in the U5 stem/loop structures upstream of the PBS (8). Secondly, deletion of the 4 consecutive A's in the U5 stem/loop of HIV-1 does not affect tRNALys3 genomic placement, either *in vitro* (131), (8) or *in vivo* (124). Since the deletion of the A-rich loop in HIV-1 does not affect $tRNA^{Lys3}$ placement, an interpretation of this data is that altering the loop to interact with the new primer tRNA may allow RT to become more processive during the initiation phase, and thus allowing the elongation phase to proceed more efficiently (8).

CHAPTER 2

EFFECT OF ALTERING THE $tRNA^{Lys3}$ CONCENTRATION IN HIV-1 UPON ITS ANNEALING TO VIRAL RNA, GAGPOL INCORPORATION AND VIRAL INFECTIVITY

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2.1 Preface

During viral assembly of HIV-1, $tRNA^{Lys}$ ($tRNA^{Lys1,2}$ and $tRNA^{Lys3}$) is selectively packaged into the virus through interactions with GagPol, where $tRNA^{Lys3}$ acts as the primer for reverse transcription. The relative ratio of $tRNA^{Lys}$ isoacceptors in the mammalian cell is approximately 2 $tRNA^{Lys3}$: 3 $tRNA^{Lys1,2}$, and this ratio is mirrored in wild-type HIV-1. Overexpression of human $tRNA^{Lys3}$ in the cytoplasm of the cell results in an increase in viral $tRNA^{Lys3}$, with a corresponding decrease in $tRNA^{Lys1,2}$, thereby keeping the amount of total viral $tRNA^{Lys}$ fixed. In this chapter, I have studied the effect of excess viral $tRNA^{Lys3}$ upon its annealing to the viral genome, and upon the infectivity of the virus, and have found there to be a direct relationship between the three, ie. the higher the concentration of viral $tRNA^{Lys3}$, the more $tRNA^{Lys3}$ genomic placement and the more infectious the virus.

LysRS, which is a $tRNA^{Lys}$-binding protein, is also selectively packaged into HIV-1, a process facilitated by Gag. In this chapter I have also shown that overexpressing LysRS in protease-negative HIV-1 results in an almost doubling of viral tRNALys, without changing the viral concentration of GagPol. Therefore it appears that LysRS plays an important role in $tRNA^{Lys}$ packaging, and that it is a limiting factor in this process, while GagPol is not.

2.2. Abstract

HIV-1 uses $tRNA^{Lys3}$ as a primer for reverse transcription, and during viral assembly, this tRNA is selectively packaged into the virus along with the other major $tRNA^{Lys}$, $tRNA^{Lys1,2}$. Increasing the cytoplasmic concentration of $tRNA^{Lys3}$ through transfection of cells with a plasmid containing both HIV-1 proviral DNA and a $tRNA^{Lys3}$ gene results in a greater incorporation of $tRNA^{Lys3}$ into virions, which is accompanied by increased annealing of $tRNA^{Lys3}$ to the viral genome and increased infectivity of the viral population. Increased viral $tRNA^{Lys3}$ is accompanied by decreased viral $tRNA^{Lys1,2}$, with the total $tRNA^{Lys}$/virion and the GagPol/Gag ratio remaining unchanged. Viral $tRNA^{Lys}$ can be doubled, with increases in both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ concentrations, by overexpressing lysyl tRNA synthetase. This also results in increased $tRNA^{Lys3}$ annealing to the viral RNA and increased viral infectivity, but again, no change in the GagPol/Gag ratio is observed. This indicates that GagPol, whose interaction is required during packaging, is not a limiting factor during $tRNA^{Lys}$ incorporation into HIV-1, and that LysRS is.

2.3. Introduction

During retroviral assembly, particular species of cellular tRNA are selectively packaged into the virus, where they are placed onto the primer binding site (PBS) of the viral genome, and are used to initiate the reverse-transcriptase-catalyzed synthesis of minus strand cDNA. The primer tRNA for members of the avian sarcoma and leukosis virus group is $tRNA^{Trp}$ (86), (110), (204), (233), (266), (279), and $tRNA^{Pro}$ for Murine Leukemia Virus (MuLV) (109), (203), (262). In mammalian cells, there are three major $tRNA^{Lys}$ isoacceptors (224). $tRNA^{Lys1,2}$, representing two $tRNA^{Lys}$ isoacceptors differing by one base pair in the anticodon stem, is the primer tRNA for several mammalian retroviruses, including Mason-Pfizer Monkey virus (MPMV) and Human Foamy Virus (HFV) , while $tRNA^{Lys3}$ is the primer tRNA for lentiviruses, including HIV-1 (162).

Selective packaging of primer tRNA is defined as an increase in the percentage of the low molecular weight RNA population representing primer tRNA in moving from the cytoplasm to the virus. For example, in Avian Myeloblastosis Virus (AMV), the relative concentration of $tRNA^{Trp}$ changes from 1.4% in the cytoplasm to 32% in the virus (266). 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% to 50-60% (176). 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 (126). 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 (266). Selective packaging of primer tRNA occurs independently of viral genomic RNA packaging in MuLV, HIV-1, and Avian Sarcoma Virus (166), (176), (207), and has been shown in HIV-1 to occur independently of Gag and GagPol processing as well (148), (176).

While it is suggestive that the selective packaging of primer tRNAs into the virion would occur in order to facilitate the annealing of the primer tRNA to the PBS through achieving higher viral concentrations of primer tRNA, experimental proof for this assumption has been absent. In avian retroviruses (95), (204) and HIV-1 (176), virions lacking functional reverse transcriptase (RT(-)) are unable to either selectively package primer tRNA or anneal it to the PBS. However, these observations do not make clear whether reduced genomic placement of primer tRNA is due to the reduction of primer tRNA in the virus or to the absence of functional RT sequences which might be required to place the tRNA on the genome. Also, RT(-) MuLV, while unable to selectively incorporate $tRNA^{Pro}$, are still capable of achieving wild-type levels of annealing of $tRNA^{Pro}$ to the PBS (95), (164), (165).

Therefore, in this work, we have artificially altered viral $tRNA^{Lys3}$ concentrations in HIV-1-transfected COS cells through expression from exogenous plasmids of either $tRNA^{Lys}$ isoacceptors or lysyl tRNA synthetase (LysRS). We find a direct correlation between viral $tRNA^{Lys3}$ concentrations, annealing to the viral RNA, and infectivity of the viral populations.

2.4. Materials and methods

2.4.1. Plasmid construction.

SVC21BH10 is a simian virus 40-based vector containing wild-type HIV-1 proviral DNA. SVC21BH10Lys3 and SVC21BH10Lys2 contain both wild-type HIV-1 proviral DNA and a human $tRNA^{Lys3}$ or $tRNA^{Lys2}$ gene, respectively. These vectors were constructed as previously described (126). SVC21BH10.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. SVC21BH10.P(-)Lys3 contains both protease negative HIV-1 proviral DNA and a human $tRNA^{Lys3}$ gene, cloned in the same way as SVC21BH10Lys3. Plasmid pM368 contains cDNA encoding full-length (1-597 amino acids) human LysRS, as previously described (251). The cDNA was PCR-amplified, and digested with EcoR1 and Xho1, whose sites were placed in each of the PCR primers. For expression in COS7 cells, the PCR DNA fragments were cloned into pcDNA 3.1 (Invitrogen) to obtain pLysRS.F, expressing full length LysRS.

2.4.2. Virus infection/transfection and purification.

COS7 cells were transfected using the calcium phosphate method as previously described (176), or, for co-transfections, with Lipofectamine (Invitrogen). Supernatant was collected 63 hours post-transfection. Viruses were pelleted from culture medium by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 1 hour. The viral pellets were then purified by centrifugation in a

Beckman SW41 rotor at 26,500 rpm for 1 hour through 15% sucrose onto a 65% sucrose cushion. The band of purified virus was removed and pelleted in 1X TNE in a Beckman Ti45 rotor at 40,000 rpm for 1 hour. Viral genomic RNA was extracted using guanidium isothiocynate, as previously described (136).

2.4.3. 1D and 2D PAGE.

Electrophoresis of ^{32}pCp-labelled viral RNA was carried out at 4°C with the Hoeffer SE620 gel electrophoresis apparatus. The gel size was 14 by 32 cm. The first dimension was run in an 11% polyacrylamide-7M urea gel for 16 hours at 800 V. After autoradiography, the piece of gel containing RNA was cut out, and run for 30 hours (25 Watt limiting); this was followed by autoradiography. All electrophoretic runs were carried out in 0.5X TBE (1X TBE is 50 mM Tris, 5mM boric acid, 1mM EDTA-Na_2). The electrophoretic gel patterns shown in this paper show only low molecular weight RNA, since the high-molecular weight viral genomic RNA cannot enter into the polyacrylamide gels. Furthermore, these patterns represent only the most abundant tRNA species present, since longer film exposures will reveal the presence of more minor-abundance species.

2.4.4. Packaging of $tRNA^{Lys3}$.

The relative amount of $tRNA^{Lys3}$ per copy of HIV-1 genomic RNA was determined by dot blot hybridization. Each sample of total viral RNA was blotted onto Hybond N+ nylon membranes (Amersham Pharmacia), and was probed with a 5′ ^{32}P-end-labelled 18-mer DNA probe specific for the 3′ end of $tRNA^{Lys3}$,

using the DNA probe: 5′-TGGCGCCCGAACAGGGAC-3′, or $tRNA^{Lys1,2}$, using the DNA probe 5′TGGCGCCCAACGTGGGGC-3′. Experiments were done in tripicate. Determination of $tRNA^{Lys}$ (i.e., both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$) used both probes together. The relative amounts of $tRNA^{Lys}$ isoacceptor per sample were analyzed using phosphor-imaging (BioRad). The blots were then stripped according to the manufacturer's instructions, and were re-probed with a 5′ ^{32}P-end-labelled 17-mer DNA probe specific for the 5′ end of HIV-1 genomic RNA, upstream of the primer binding site (5′-CTGACGCTCTCGCACCC-3′). Phosphor-imaging was used to quantitate the relative amount of HIV-1 genomic RNA per sample, and the relative amount of $tRNA^{Lys3}$ or $tRNA^{Lys1,2}$ per copy of HIV-1 genomic RNA was determined. The amount of total viral RNA used in these determinations contained 3-10X10^8 copies genomic RNA, an amount producing signals within the linear range of measurement for hybridization of both $tRNA^{Lys}$ isoacceptors and genomic RNA, as shown by standard curves generated by using a dilution series of total viral RNA which is hybridized with the DNA probes complementary to either $tRNA^{Lys}$ or genomic RNA.

2.4.5. Primer extension.

$tRNA^{Lys3}$–primed initiation of reverse transcription was measured by the ability of $tRNA^{Lys3}$ to be extended by 6 bases in an *in vitro* HIV-1 reverse transcription reaction. Total viral RNA was used as the source of primer tRNA/template. Each sample contained 5 x 10^8 copies of genomic RNA, measured as previously described (126), and produces signals falling within the

linear range of measurement, as shown by generating a standard cruve using a dilution series of total BH10 viral RNA as the source of primer/template. The sequence of the first 6 deoxynucleoside triphosphates incorporated is CTGCTA. The reactions were carried out in a volume of 20 μL containing 50 mM Tris-HCl (pH 7.8), 100 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.2 mM dCTP, 0.2 mM dTTP, 5 μCi α-^{32}P-dGTP and 0.05mM ddATP (instead of dATP, thereby terminating the reaction at 6 bases), 50 ng HIV-1 RT, and RNase inhibitor (Amersham Pharmacia). After incubation for 15 minutes at 37°C, the samples were precipitated with isopropanol, and were electrophoresed in a 6% polyacrylamide gel at 70 W for 1.5 hours. Relative amounts of $tRNA^{Lys3}$ placement were analyzed by comparing the intensity of bands with phosphor-imaging.

2.4.6. Statistical analysis of dot blots and primer extension.

All analyses were done in triplicate, with triplicate samples in each experiment. The statistical analyses employed herein include column statistics and one-way analysis of variance (ANOVA). The lowest level of significance was set at $p < 0.05$.

2.4.7. Viral infectivity.

Viral infectivity was measured by the MAGI assay (150). MAGI cells are CD4+ HeLa cells containing an HIV-1 LTR fused to a ß-galactosidase reporter gene. A total of 4×10^4 cells per well were cultured in 1 mL of media, in 24-well plates. After 24 hours, the media was removed and was replaced with 150 μL of

culture medium containing various dilutions of virus. DEAE-Dextran was added to a final concentration of 20 μg/ml, and viral absorption took place for 2 hours, after which 1 mL of fresh culture medium was added. 48 hours later, the medium was removed and fixative (1% formaldehyde, 0.2% gluteraldehyde in PBS) was added for 5 minutes. The fixative was removed and 200 μL of staining solution was added (for 1 mL: 950 μL PBS, 20 μL of 0.2 M potassium ferrocyanide, 20 μL of 0.2 M potassium ferricyanide, 1.0 μL of 2 M $MgCl_2$, and 10 μL of X-gal stock [stock = 40 mg/mL in DMSO]). The cells were washed twice with PBS and the number of blue cells per well per equal amount of p24 were counted. Only wells containing 20 to 100 blue cells were analyzed, keeping within linear range of analysis (150).

2.4.8. Protein analysis.

Viral particles were purified as described above, and viral proteins were extracted with RIPA buffer (10 mM Tris, pH 7.4, 100mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP40, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin A, 100 mg/ml PMSF). The viral lysates were analyzed by SDS PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amersham Pharmacia). Detection of protein by Western blotting utilized monoclonal antibodies that are specifically reactive with HIV-1 capsid (Zepto Metrocs Inc.), reverse transcriptase (NIH-AIDS Research and Reference Reagent Program) and a polyclonal antibody for human lysyl tRNA synthetase (a kind gift from Kiyotaka Shiba, Japan). Detection of HIV proteins was performed

by enhanced chemiluminescence (NEN Life Sciences Products) using as secondary antibodies anti-mouse (for capsid and reverse transcriptase) and anti-rabbit (lysyl tRNA synthetase), both obtained from Amersham Life Sciences.

2.5. Results

2.5.1. Overexpression of $tRNA^{Lys}$ isoacceptors from exogenous plasmids.

We have previously shown that viral $tRNA^{Lys3}$ content can be increased by transfecting COS7 cells with an SV40-based plasmid containing both the HIV-1 proviral DNA and a human $tRNA^{Lys3}$ gene (BH10Lys3), and that as a result, $tRNA^{Lys1,2}$ packaging into the virus decreases (126). We have also in a similar manner produced viruses with an excess of $tRNA^{Lys2}$ and a decrease in viral $tRNA^{Lys3}$ (BH10Lys2), by transfecting COS7 cells with a plasmid containing the HIV-1 proviral DNA and a human gene for $tRNA^{Lys2}$ (obtained from Dr Robert M. Pirtle, University of North Texas). The inverse relationship between viral concentrations of $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ can be seen in Figure 2.1. The data in Figure 2.1A shows the 2 dimension polyacrylamide gel electrophoresis (2D PAGE) patterns of low molecular weight viral RNA in wild-type HIV-1 (BH10), BH10Lys3, and BH10Lys2. The identity of the $tRNA^{Lys}$ isoacceptors found in spots 1 and 2 ($tRNA^{Lys1,\ 2}$), and spot 3 ($tRNA^{Lys3}$) have been previously determined (136). BH10Lys3 has an additional small dark spot (3′) which has been identified as an additional $tRNA^{Lys3}$ species by a partial T1 digestion pattern (data not shown) which is identical to the partial T1 digestion pattern of the major $tRNA^{Lys3}$ spot (136). This species can sometimes be seen as a very light spot in wild-type virus.

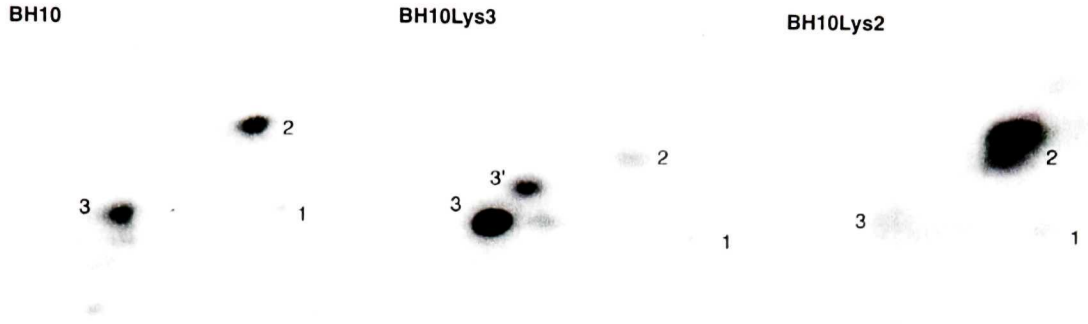
In Figure 2.1B, the relative amount of $tRNA^{Lys}$/virion was determined in the three types of virions, using hybridization probes for both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ to determine the relative amount of $tRNA^{Lys}$/genomic RNA. The experimental

Figure 2.1. Alteration of $tRNA^{Lys}$ in HIV-1 as a result of overexpression of $tRNA^{Lys3}$ or $tRNA^{Lys1,2}$. Wild-type viruses were produced from COS7 cells transfected with BH10, BH10Lys3, or BH10Lys2, and total viral RNA was extracted.

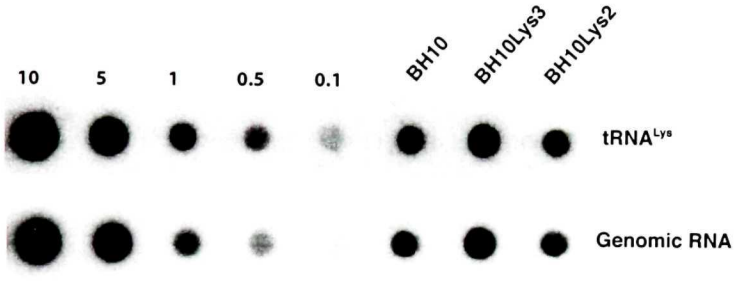
A. 2D PAGE analysis of low molecular weight viral RNA. Total viral RNA was 3′ end-labeled with ^{32}pCp, and electrophoresed in 11% polyacrylamide in the first dimension, and 20% polyacrylamide in the second dimension. Only low molecular weight RNA moves into the gel, and is detected by autoradiography. Spots 3,3′, $tRNA^{Lys3}$; spots 1 and 2, $tRNA^{Lys1,2}$ (Partial sequencing of these two spots does not distinguish between $tRNA^{Lys1}$ and $tRNA^{Lys2}$, which differ by only 1 base pair in the anticodon stem). BH10: Wild-type HIV-1 produced from cells transfected with a plasmid containing wild-type HIV-1 proviral DNA. BH10Lys3: HIV-1 produced from cells transfected with a plasmid containing both HIV-1 proviral DNA and a human gene for $tRNA^{Lys3}$. BH10Lys2: HIV-1 produced from cells transfected with a plasmid containing both HIV-1 proviral DNA and a human gene for $tRNA^{Lys2}$. The pattern indicates that $tRNA^{Lys2}$ is found in spot 2, and not spot 1.

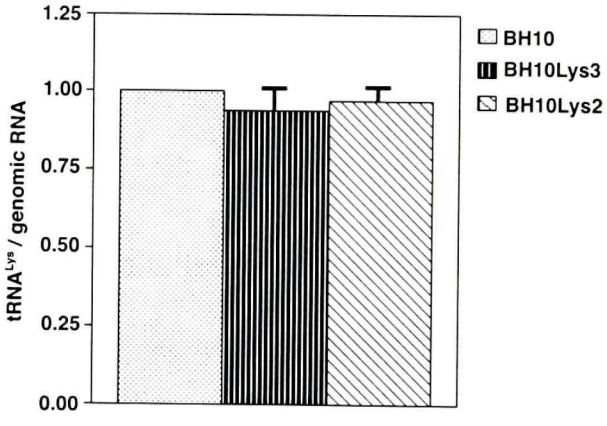
B. Analysis of the viral concentrations of $tRNA^{Lys}$. Dot-blots of viral RNA (containing 3-10 x 10^8 copies genomic RNA) were hybridized with DNA probes complementary to both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$, or to viral genomic RNA. Hybridization signals were analyzed by phosphorimaging, and the ratio of $tRNA^{Lys}$ (both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$)/genomic RNA was determined for virions produced from cells transfected with BH10, BH10Lys3, or BH10Lys2. The standard curves shown in the left part of the blot in panel B contain a dilution series of BH10 viral RNA, hybridized with the DNA probes complementary to either $tRNA^{Lys}$ or genomic RNA. Statistical analyses employed herein include column statistics and one-way analysis of variance (ANOVA), where $n=3$, $p < 0.05$.

A


BH10
BH10Lys3
BH10Lys2
2
3
1
3'
2
3
1
2
3
1

B


10
5
1
0.5
0.1
BH10
BH10Lys3
BH10Lys2
tRNA^{Lys}
Genomic RNA


1.25
1.00
0.75
0.50
0.25
0.00
tRNA^{Lys} / genomic RNA
BH10
BH10Lys3
BH10Lys2

data is shown in Figure 2.1B, and indicates little change in the total $tRNA^{Lys}$/virion.

This conclusion is supported by the data in Figure 2.2A. In these experiments, the relative concentration of $tRNA^{Lys3}$/genomic RNA, normalized to wild-type, was determined by hybridizing dot blots of total viral RNA with DNA probes specific for $tRNA^{Lys3}$ and for genomic RNA, and the values obtained are graphed in Figure 2.2A. BH10Lys3 has approximately 1.6 times more $tRNA^{Lys3}$ than wild-type, while BH10Lys2 has less than one fifth the amount of $tRNA^{Lys3}$ found in wild-type virions. While the $tRNA^{Lys}$ species that decreases can often not be seen by 2D PAGE (see Figure 2.1), it is quite measurable by hybridization. Thus, the ^{32}pCp ligation reaction used to label the $tRNA^{Lys}$ species detected in 2D PAGE appears to show a greater insensitivity to lower amounts of viral $tRNA^{Lys}$ than does the hybridization reaction.

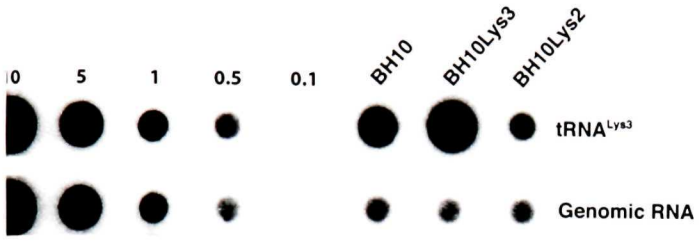
We next investigated in these three viral preparations whether the amount of $tRNA^{Lys3}$ packaged into the virus influences the amount of extendable $tRNA^{Lys3}$ placed onto the primer binding site (PBS). The first 6 bases incorporated into DNA during the initiation of reverse transcription are CTGCTA. $tRNA^{Lys3}$ extension was measured in an *in vitro* reaction using equal amounts of total viral RNA as the source of primer $tRNA^{Lys3}$/ genomic RNA template, exogenous HIV-1 RT, dCTP, dTTP, α-^{32}P-dGTP, and ddATP. This will result in a six base DNA extension of the $tRNA^{Lys3}$, and the amount of DNA extension/genomic RNA can be determined on 1D-PAGE, as shown in Figure 2.2B. Here, relative signal intensities were measured by phosphor-imaging, with the results being graphed.

Figure 2.2. Effect of $tRNA^{Lys3}$ concentrations in wild-type virions upon $tRNA^{Lys3}$ annealing and viral infectivity. Wild-type viruses were produced from COS7 cells transfected with BH10, BH10Lys3, or BH10Lys2.

A. Total viral RNA was extracted, and dot-blots of viral RNA were hybridized with DNA probes complementary to either $tRNA^{Lys3}$ or viral genomic RNA. Hybridization signals were analyzed by phosphorimaging, and the ratio of $tRNA^{Lys3}$/genomic RNA was determined for virions produced from cells transfected with BH10, BH10Lys3, or BH10Lys2. The standard curves shown in the left part of the blots were generated as described for Figure 2.1.

B. $tRNA^{Lys3}$ annealing to viral RNA. Total viral RNA was extracted, and used as the source of primer $tRNA^{Lys3}$/genomic RNA template in an *in vitro* reverse transcription reaction, carried out in the presence of α-^{32}P-dGTP, dCTP, dTTP, and ddATP. This will result in a 6 base extension product since the first 6 bases incorporated are CTGCTA. Products were analyzed by 1D PAGE, using samples containing equal amounts of genomic RNA. The standard curve shown in the left part of the blot in panel B contains a dilution series of total BH10 viral RNA, which is used as the source of primer/template. Statistical analyses employed in panels A and B include column statistics and one-way analysis of variance (ANOVA), where $n=3$, $p < 0.05$. A "*" indictes statistically significant differences.

C. Viral infectivity. Infectivity was determined using the MAGI assay as described in the text.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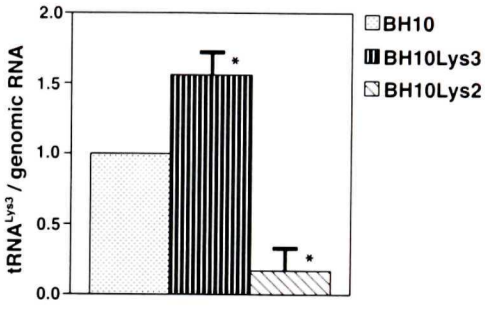
BH10Lys3
BH10Lys2
tRNA^{Lys3}
Genomic RNA


2.0
1.5
1.0
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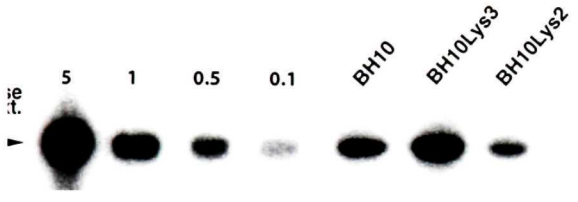
0.0
tRNA^{Lys3} / genomic RNA
*
*
BH10
BH10Lys3
BH10Lys2


5
1
0.5
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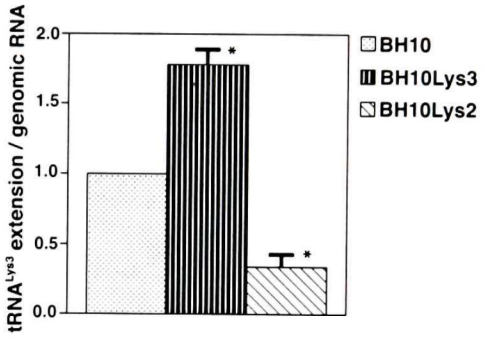
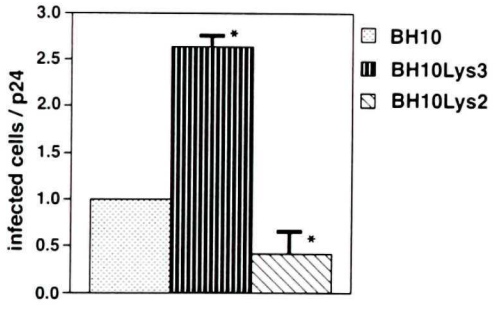
BH10
BH10Lys3
BH10Lys2


2.0
1.5
1.0
0.5
0.0
tRNA^{Lys3} extension / genomic RNA
*
*
BH10
BH10Lys3
BH10Lys2


3.0
2.5
2.0
1.5
1.0
0.5
0.0
infected cells / p24
*
*
BH10
BH10Lys3
BH10Lys2

This data indicates a correlation between $tRNA^{Lys3}$ incorporated into the viruses and the amount of extendable $tRNA^{Lys3}$ placed onto the PBS.

The relative infectivity of the three viral preparations was also measured using the MAGI assay (150), which measures single round infectivity. CD4-positive HeLa cells containing the ß-galactosidase gene fused to the HIV-1 LTR are infected with virus. Cells infected with HIV-1 will have the ß-galactosidase gene expressed, and such cells can be detected using an appropriate substrate for the enzyme, such as X-gal, whose metabolism turns the cells blue. The number of blue cells is a measure of viral infectivity. The relative infectivity of the different viral populations is graphed in Figure 2.2C, and indicate that infectivity is directly correlated with $tRNA^{Lys3}$ packaging and annealing of extendable $tRNA^{Lys3}$ onto the PBS.

The increase in $tRNA^{Lys3}$/virus does not result in any increase in GagPol incorporation. This was studied in protease-negative virions to facilitate detection of the GagPol precursor. Figure 2.3A shows the 2D PAGE patterns of low molecular weight viral RNA in protease-negative BH10.P(-) and BH10.P(-)Lys3 virions. The patterns are nearly identical to their protease-positive counterparts, and as shown in these gels and in the table in panel C, the increase in $tRNA^{Lys3}$ is accompanied by a decrease in $tRNA^{Lys1,2}$, with the total $tRNA^{Lys}$/virion remaining the same. In Figure 2.3B, western blots of viral lysates probed with anti-CA and anti-RT indicate little change in the GagPol:Gag ratios (listed in the table in panel C).

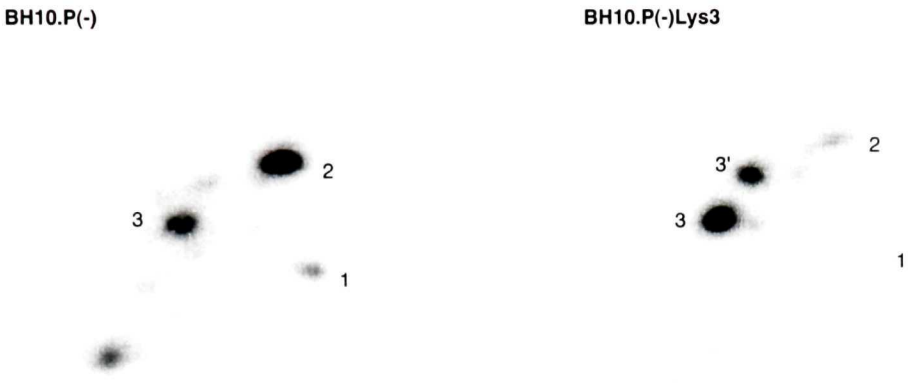
Figure 2.3. Alteration of $tRNA^{Lys3}$ in protease-negative HIV-1 as a result of overexpression of $tRNA^{Lys3}$. Protease-negative viruses were produced from COS7 cells transfected with BH10.P(-) or BH10.P(-)Lys3.

A. 2D PAGE analysis of low molecular weight viral RNA. Total viral RNA was 3′ end-labeled with ^{32}pCp, and electrophoresed. Conditions for 2D PAGE and labeling of spots is as described in Figure 2.2.A.

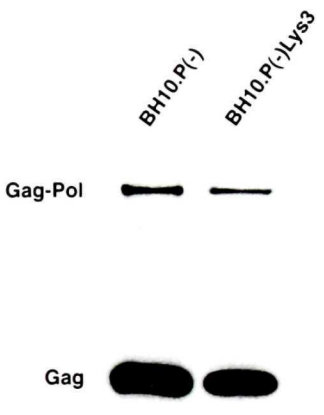
B. Western blots of viral lysates, probed with anti-CA and anti-RT. The results, quantitated by phosphorimaging, are listed in panel C as the GagPol:Gag ratios.

C. Incorporation of $tRNA^{Lys}$ into HIV-1. Dot-blots of viral RNA were hybridized with DNA probes complementary to $tRNA^{Lys3}$ alone, $tRNA^{Lys}$ (both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$), and to viral genomic RNA. The results were quantitated by phosphorimaging, and the $tRNA^{Lys3}$/genomic RNA or $tRNA^{Lys}$/genomic RNA ratios are listed in panel C.

A



B



C

Ratio*	BH10.P(-)	BH10.P(-)Lys3
$tRNA^{Lys3}$: Genomic RNA	1.0	1.9
$tRNA^{Lys}$: Genomic RNA	1.0	1.0
Gag-Pol : Gag	1.0	0.9

*normalized to BH10.P(-)

2.5.2. Overexpression of LysRS from an exogenous plasmid.

The stable viral concentration of $tRNA^{Lys}$ during overexpression of $tRNA^{Lys3}$ or $tRNA^{Lys2}$ indicates that there is some factor limiting the incorporation of $tRNA^{Lys}$ isoacceptors. Although the incorporation of GagPol into viral particles does not change when $tRNA^{Lys3}$ packaging is increased, this could reflect the fact that the total $tRNA^{Lys}$ packaged doesn't change. We have, however, recently found that overexpression of LysRS in protease-negative virions can result in up to a two fold increase in both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ in the virions (36). Thus, LysRS could be the limiting factor, but its expression might also stimulate GagPol incorporation. We have therefore investigated the effect of overexpression of LysRS upon both $tRNA^{Lys3}$ annealing to the PBS and upon GagPol incorporation. COS7 cells were cotransfected with both protease-negative HIV-1 proviral DNA BH10.P(-) and a plasmid encoding human LysRS, pLysRS.F. We have previously shown that this results in greater incorporation of both LysRS and $tRNA^{Lys}$ into virions (36), and Figure 2.4A shows that unlike overexpression of $tRNA^{Lys3}$, overexpression of LysRS results in increases in both $tRNA^{Lys3}$/genomic RNA and $tRNA^{Lys1,2}$/genomic RNA, resulting in a significant increase in $tRNA^{Lys}$ in the virion. Figure 2.4B shows that, like overexpression of $tRNA^{Lys3}$, overexpression of LysRS results in an increase in annealing of $tRNA^{Lys3}$ to the viral RNA, as determined using total viral RNA isolated from the two types of virions as the source of primer/template in the 6 base *in vitro* RT extension reaction. This provides further proof that $tRNA^{Lys3}$ annealing is proportional to the amount of $tRNA^{Lys3}$ packaged into the virions.

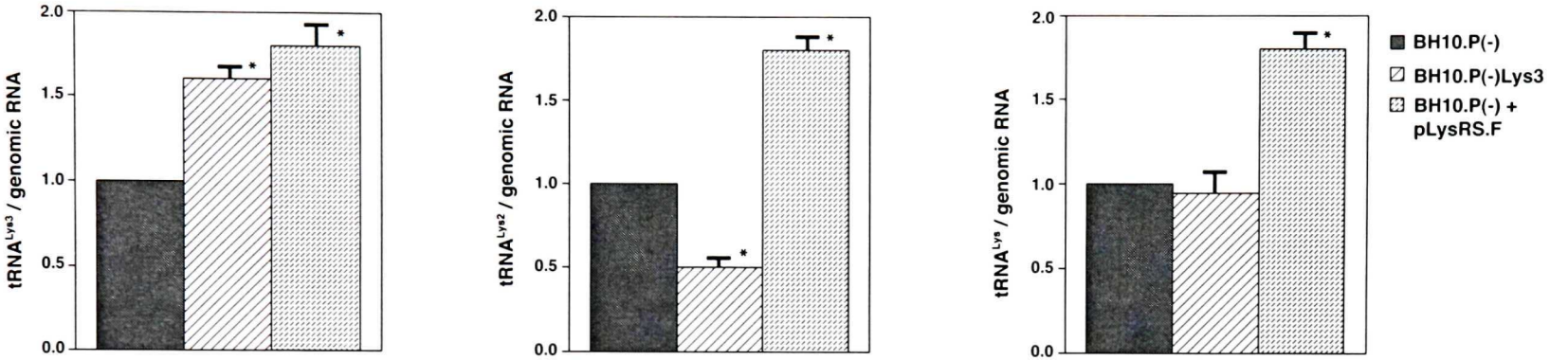
Figure 2.4. The effect of overexpression of LysRS upon $tRNA^{Lys}$ incorporation and annealing, GagPol incorporation, and viral infectivity. Protease-negative viruses were produced from COS7 cells transfected with BH10.P(-), BH10.P(-)Lys3, or cotransfected with BH10.P(-) and pLysRS.F.

A. $tRNA^{Lys}$ incorporation. Dot-blots of viral RNA were hybridized with DNA probes complementary to either $tRNA^{Lys3}$ or $tRNA^{Lys1,2}$ alone, $tRNA^{Lys}$ (both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$), and to viral genomic RNA. The results were quantitated by phosphorimaging, and the ratios of $tRNA^{Lys3}$, $tRNA^{Lys1,2}$, or $tRNA^{Lys}$, to genomic RNA are plotted for the three viral types. Statistical analyses of the results are as described in the legend to Figure 2.1.

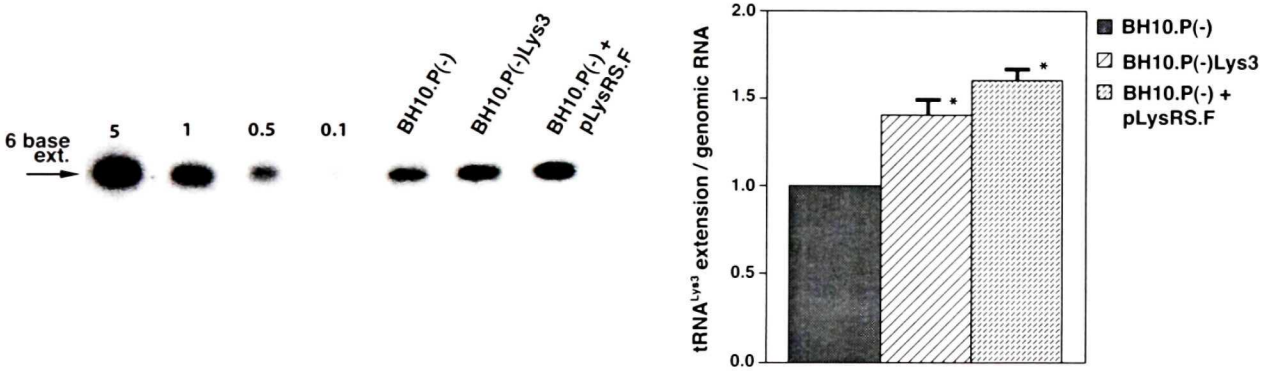
B. $tRNA^{Lys3}$ annealing to viral RNA. Total viral RNA was extracted, and used as the source of primer $tRNA^{Lys3}$/genomic RNA template in an *in vitro* reverse transcription reaction, as described for Figure 1B. Products were analyzed by 1D PAGE, using samples containing equal amounts of genomic RNA. Generation of the standard curve and statistical analyses of the results are as described in the legend to Figure 2.2.B.

C. Western blots of viral lysates, probed with anti-CA and anti-RT. The results, quantitated by phosphorimaging, are listed as the GagPol:Gag ratios beneath each lane.

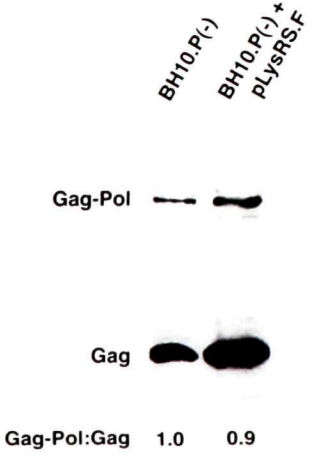
A


tRNA^Lys3 / genomic RNA
tRNA^Lys2 / genomic RNA
tRNA^Lys / genomic RNA
2.0
1.5
1.0
0.5
0.0
*
BH10.P(-)
BH10.P(-)Lys3
BH10.P(-) + pLysRS.F

B


6 base ext.
5
1
0.5
0.1
BH10.P(-)
BH10.P(-)Lys3
BH10.P(-) + pLysRS.F
tRNA^Lys3 extension / genomic RNA
2.0
1.5
1.0
0.5
0.0
*
BH10.P(-)
BH10.P(-)Lys3
BH10.P(-) + pLysRS.F

C


BH10.P(-)
BH10.P(-) + pLysRS.F
Gag-Pol
Gag
Gag-Pol:Gag
1.0
0.9

The Western blot shown in Figure 2.4C shows that the GagPol:Gag ratio in either type of virion remains similar, even though $tRNA^{Lys}$/genomic RNA has significantly increased. This indicates that during packaging of $tRNA^{Lys}$, GagPol is in excess, and LysRS is limiting.

2.6. Discussion

The work herein indicates a direct relationship between $tRNA^{Lys3}$ incorporated into the viral population, $tRNA^{Lys3}$-primed initiation of reverse transcription, and infectivity of the viral population. The viral population may contain viruses which have packaged primer $tRNA^{Lys3}$ to different extents, with resulting different degrees of $tRNA^{Lys3}$ annealing and viral infectivity. This variability could be the result of mutations in viral or cellular proteins involved in this process, but could also result from random differences in the amount of GagPol or LysRS incorporated into each virion during assembly. The evolution of a biochemical mechanism for enriching $tRNA^{Lys3}$ incorporation might help reduce the variability of packaging into viruses.

The increase in $tRNA^{Lys3}$ annealing resulting from overexpression of $tRNA^{Lys3}$ or LysRS is not accompanied by a change in the GagPol:Gag ratio, implying no increase in the incorporation of GagPol (assuming that Gag incorporation has not changed). This result implies that LysRS, rather than GagPol, is a limiting factor for $tRNA^{Lys}$ packaging, and explains why total viral $tRNA^{Lys}$ remains constant when one of the $tRNA^{Lys}$ isoacceptors is overexpressed.

Seeming to stand counter to the claim of the importance of selective packaging of primer tRNA in virions for obtaining optimum annealing of primer tRNA to the viral template is the work from C.D. Morrow's laboratory. This work indicates that some non-lysyl tRNAs, such as $tRNA^{His}$ (280) and $tRNA^{Meti}$ (140), can be used as primer tRNAs without their selective packaging. For example,

changing the $tRNA^{Lys3}$-complementary PBS to a sequence complementary to $tRNA^{His}$ does not allow the stable use of $tRNA^{His}$ as a primer, and the PBS reverts back to being complementary to $tRNA^{Lys3}$ (265). However, if in addition to the PBS, the A-rich loop just upstream of the PBS is also made complementary to the anticodon loop of $tRNA^{His}$, $tRNA^{His}$ will be stably used as a primer tRNA, although some other point mutations in the regions near the PBS also occur to facilitate this (280). This mutant virion, termed His-AC-GAC, continues to selectively package $tRNA^{Lys}$, but does not appear to selectively package the new primer tRNA, $tRNA^{His}$ (280). Although the replication kinetics of this virus is generally lower than the wild-type virion using $tRNA^{Lys3}$ as a primer (280), replication rates of 50% wild-type without selective packaging of $tRNA^{His}$ lead to the conclusion that selective packaging of primer tRNA is not required in the mutant HIV-1. A more general conclusion that might be derived from these observations is that the virus, put under certain conditions requiring the use of a new primer tRNA, can find new ways to utilize low concentrations of the viral tRNA more easily than it can find new ways to selectively package this tRNA. In fact, the normal annealing of $tRNA^{Pro}$ to the viral RNA in MuLV does not require selective packaging of this primer (266), and wild type levels of $tRNA^{Pro}$ annealing are found in RT-negative MuLV in which any selective packaging that might occur is abolished (95), (164), (165). Thus, while there may be alternative ways in which retroviruses can develop to achieve optimum primer tRNA annealing to the PBS, wild-type HIV-1 and avian retroviruses appear to achieve this through selective packaging of primer tRNAs into the viruses.

CHAPTER 3

OVEREXPRESSION OF $tRNA^{LYS}$ OR LYSYL tRNA SYNTHETASE RESULTS IN AN INCREASE IN REVERSE TRANSCRIPTASE AND INTEGRASE IN HIV-1

This chapter was adapted from a manuscript to be submitted to the Journal of Virology.

3.1. Preface

The control of the proteolytic processing of Gag and GagPol is an important aspect of the life cycle in HIV-1. Premature activation of the viral protease could have disasterous effects, including the processing of precursor proteins before viral budding, which, among other things, could result in the loss of both membrane-binding and non-membrane binding viral proteins to the cytoplasm of the cell, and giving rise to a viral population with reduced infectivity. It would seem likely that controls are in place to prevent such premature processing from taking place.

There is evidence in this next chapter that such a control exists. We have found that, under conditions where $tRNA^{Lys}$ or LysRS are enriched in the cytoplasm of the cell, there is an increase of viral packaging of RT and IN, yet there is no evidence for an increase in viral GagPol, the precursor for RT and IN. This chapter, therefore, provides additional evidence to Chapter 2 in supporting the fact that a high viral $tRNA^{Lys}$/LysRS content is beneficial to the virus.

3.2. Abstract

In HIV-1, premature processing of Gag and GagPol by viral protease may be inhibited by the presence of these proteins within a $tRNA^{Lys}$ packaging complex composed of genomic RNA, Gag, GagPol, $tRNA^{Lys}$, and lysyl tRNA synthetase (LysRS). Overexpression of either $tRNA^{Lys2}$ or $tRNA^{Lys3}$ in the cell results in an increase in viral reverse transcriptase (RT) and integrase (IN), but no corresponding increase in GagPol incorporation is seen in protease-negative viruses, suggesting an increased retention of processed RT and IN in the virion. Under these conditions, viral $tRNA^{Lys}$ does not increase, but viral LysRS does, indicating that LysRS may be responsible for the increased retention of RT and IN. Overexpression of LysRS in the cell results in an increase in the incorporation of both LysRS and $tRNA^{Lys}$ into the viruses, with a concomitant increase in viral RT and IN, but again, no increase in GagPol incorporation is seen in protease-negative viruses, suggesting that GagPol is not limiting for $tRNA^{Lys}$ incorporation. We postulate that increased LysRS packaging may inhibit premature processing of Gag and GagPol, ensuring the encapsidation of the maximum amount of RT and IN in the virus after processing.

3.3. Introduction

During viral assembly, the Gag and GagPol precursors are processed into the final mature viral proteins by the viral protease located within GagPol (248). This process is believed to occur during or immediately after viral budding (143), and mechanisms which prevent premature processing of the viral precursors may be required to prevent loss of processed products before budding is complete. Since protease dimerization is required for the enzyme's activity (248), the concentration of GagPol at the plasma membrane could be required to favor dimerization. Another regulatory mechanism could be a conformation of GagPol during its transit to the plasma membrane making it resistant to dimerization or protease activation. Such a GagPol transport complex probably includes Gag, since interaction with Gag is required for GagPol incorporation into budding viruses (198), (235), and viral genomic RNA, which is required for Gag multimerization (14).

Addition components of the complex probably also include $tRNA^{Lys}$ and lysyl tRNA synthetase (LysRS), the enzyme which aminoacylates $tRNA^{Lys}$. During HIV-1 assembly, the $tRNA^{Lys}$ isoacceptors, $tRNA^{Lys3}$ (the primer tRNA for the reverse-transcriptase catalyzed synthesis of minus strand strong stop cDNA (178), and $tRNA^{Lys1,2}$, are selectively packaged into HIV-1 (136), and this incorporation requires the presence of GagPol (176). LysRS is also selectively packaged into the virus (35). These results suggest that LysRS may act as a signal for targeting the interaction of $tRNA^{Lys}$ with viral proteins. Since GagPol interacts with both Gag (198), (235) and $tRNA^{Lys}$ (148), and only Gag is required

among viral proteins for the incorporation of LysRS into Gag viral-like particles (VLP's) (35), the incorporation of $tRNA^{Lys}$ into HIV-1 may involve an interaction of Gag/GagPol with $tRNA^{Lys}$/LysRS, with Gag interacting with LysRS and GagPol interacting with $tRNA^{Lys}$.

GagPol in this transport complex may be more resistant to either dimerization or protease activation than when found in budding virions. In this report, we show that overexpression of each of three of these components, $tRNA^{Lys3}$, $tRNA^{Lys2}$, or LysRS, results in an increase of RT and IN in the virion. This overexpression does not result in an increase in GagPol incorporation in protease-negative viruses, suggesting that the increase in viral RT and IN results from increased retention of the processed products, and also indicates that GagPol is not an immediate limiting factor for LysRS or $tRNA^{Lys}$ incorporation into virions.

3.4. Materials and Methods

3.4.1. Plasmid construction.

SVC21BH10 is a simian virus 40-based vector containing wild-type HIV-1 proviral DNA. SVC21BH10Lys3 and SVC21BH10Lys2 contain both wild-type HIV-1 proviral DNA and a human $tRNA^{Lys3}$ or $tRNA^{Lys2}$ gene, respectively. These vectors were constructed as previously described (126). SVC21BH10.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. SVC21BH10.P(-)Lys3 contains both protease negative HIV-1 proviral DNA and a human $tRNA^{Lys3}$ gene, cloned in the same way as SVC21BH10Lys3. pLysRS.F contains a gene for full length LysRS, cloned into pCDNA3.1 (Invitrogen); its construction was previously described (98).

3.4.2. Virus infection/transfection and purification.

COS7 cells were transfected using the calcium phosphate method as previously described (176), or, for co-transfections, with Lipofectamine (Invitrogen). Supernatant was collected 63 hours post-transfection. Virus was pelleted from culture medium by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 1 hour. The viral pellets were then purified by centrifugation in a Beckman SW41 rotor at 26,500 rpm for 1 hour through 15% sucrose onto a 65% sucrose cushion. The band of purified virus was removed and pelleted in 1X TNE in a Beckman Ti45 rotor at 40,000 rpm for 1 hour. Viral proteins were extracted with RIPA buffer (10 mM Tris, pH 7.4, 100mM NaCl, 1% sodium

deoxycholate, 0.1% SDS, 1% NP40, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin A, 100 mg/ml PMSF).

3.4.3. Protein analysis

The viral lysates were analyzed by SDS PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amersham Pharmacia). Detection of protein by Western blotting utilized monoclonal antibodies that are specifically reactive with HIV-1 capsid (Zepto Metrocs Inc.), reverse transcriptase (NIH AIDS Reagent Program), and rabbit polyclonal antibody to human lysyl tRNA synthetase (a kind gift from Dr. Kiyotaka Shiba, Japan). Detection of HIV proteins was performed by enhanced chemiluminescence (NEN Life Sciences Products) using sheep anti-mouse (anti-RT and anti-capsid) or donkey anti-rabbit (anti-lysyl synthetase) as a secondary antibody (Amersham Life Sciences). Relative signal intensities were quantified using NIH-1.67 imaging software.

3.5. Results

3.5.1. Overexpression of $tRNA^{Lys}$ or LysRS increases the viral concentration of RT and IN.

COS7 cells were transfected with a plasmid containing HIV-1 proviral DNA and a gene for either $tRNA^{Lys3}$ (BH10Lys3) or $tRNA^{Lys2}$ (BH10Lys2). Previous studies have shown that overexpression of either $tRNA^{Lys}$ isoacceptor results in an increase in packaging of that isoacceptor into the virion, with a corresponding decrease in the viral concentration of the other isoacceptor, i.e., the total viral $tRNA^{Lys}$/virion remains constant (98), (126). Viruses were purified, and Western blots of viral protein were probed first with anti-RT (Figure 3.1A, upper blot), stripped, and reprobed with anti-CA (Figure 3.1A, lower blot). Detection of RT was in the linear range, as indicated by a Western blot of increasing amounts of purified RT (Figure 3.1B). Phosphorimaging analysis indicated that there was approximately 1.7 times more RT/CA in virions produced from BH10Lys3 and BH10Lys2 than in wild-type virus.

As shown in Figure 3.2, the viral concentration of IN is also increased upon overexpression of a $tRNA^{Lys}$ gene. In the experiment shown, overexpression of $tRNA^{Lys3}$ results in a 2.7 fold increase in the RT/CA compared to that found in wild type virions. After stripping the Western blot, and reprobing with anti-IN, we see that IN increases in a similar fashion. Similar results are seen when $tRNA^{Lys1,2}$ is overexpressed (data not shown).This increase of viral RT and IN as a result of overexpression of either $tRNA^{Lys}$ isoacceptor is not associated with an increase in the viral concentration of total $tRNA^{Lys}$, nor is it

Figure 3.1. Content of reverse transcriptase in HIV-1 containing increased amounts of $tRNA^{Lys3}$ or $tRNA^{Lys2}$.

A. Western blots of viral lysates produced from cells transfected with BH10, BH10Lys3, or BH10Lys2. Blots were probed with anti-RT and anti-CA. Bands were quantitated by phosphorimaging, and RT/CA ratios are listed below the lanes.

B. Western blots containing increasing amount of purified HIV-1 RT, probed with anti-RT.

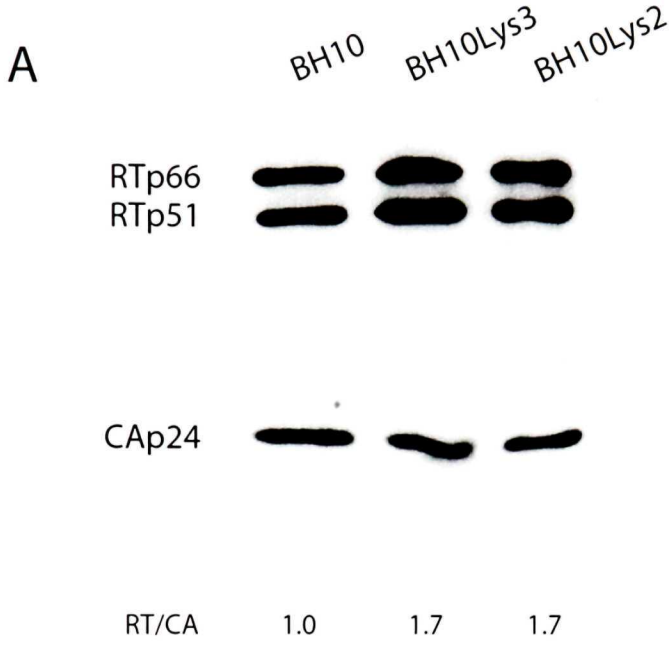
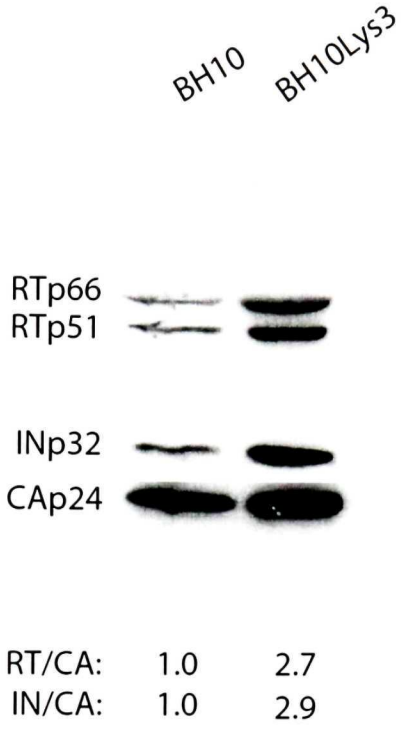

A
BH10
BH10Lys3
BH10Lys2
RTp66
RTp51
CAp24
RT/CA
1.0
1.7
1.7
B
1
2
4
8
16
32
64

Figure 3.2. Content of reverse transcriptase and integrase in HIV-1.
Anti-IN was directed against either the first 16 amino acids of integrase (NIH AIDS Research and Reference Reagent Program) or amino acids 276-288 of integrase (a gift from Dr. Mark Muesing, Aaron Diamond AIDS Research Center). Bands were quantitated by phosphorimaging, and RT/CA and IN/CA ratios are listed below the lanes.


BH10
BH10Lys3
RTp66
RTp51
INp32
CAp24
RT/CA: 1.0 2.7
IN/CA: 1.0 2.9

associated with an increase in the incorporation of GagPol (98) (126).This suggests that some other molecule may be responsible for the increase of viral RT and IN.

3.5.2. Role of LysRS in increasing viral RT and IN.

Since LysRS is also selectively packaged into HIV-1 during assembly (35), and may be part of a $tRNA^{Lys}$ packaging complex which could contain Gag/GagPol complexed with $tRNA^{Lys}$/LysRS, an increase in its concentration in the virus could be associated with the increase in RT and IN. In Figure 3.3, we show that the overexpression of $tRNA^{Lys3}$ does in fact result in an increased concentration of viral LysRS. COS7 cells were transfected with either BH10 or BH10Lys3, and Western blots of viral proteins were probed first with anti-LysRS, and then with anti-CA (Figure 3.3). Overexpression of $tRNA^{Lys3}$ not only results in an increase in RT and IN, but also results in a similar increase in the viral concentration of LysRS.

Direct overexpression of LysRS also increases the viral concentration of RT and IN, and these results are shown in Figure 3.4. COS7 cells were transfected with either a plasmid containing BH10 alone, or cotransfected with this plasmid plus a plasmid containing the human LysRS gene (LysRS.F). Western blots of viral protein were probed consecutively with anti-RT (upper), anti-IN (middle), and anti-CA (lower). The results, analyzed by phosphorimaging, indicate that overexpression of LysRS again results in an increase in the viral concentration of RT and IN.

Figure 3.3. Content of LysRS in HIV-1.

Western blots were probed with anti-CA and anti-LysRS (a kind gift from Kiyotaka Shiba, Japan). Bands were quantitated by phosphorimaging, and LysRS/CA ratios are listed below the lanes.

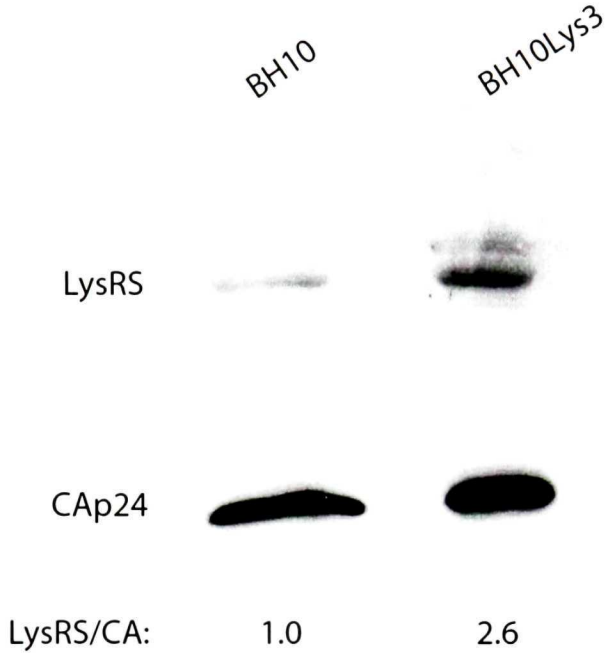
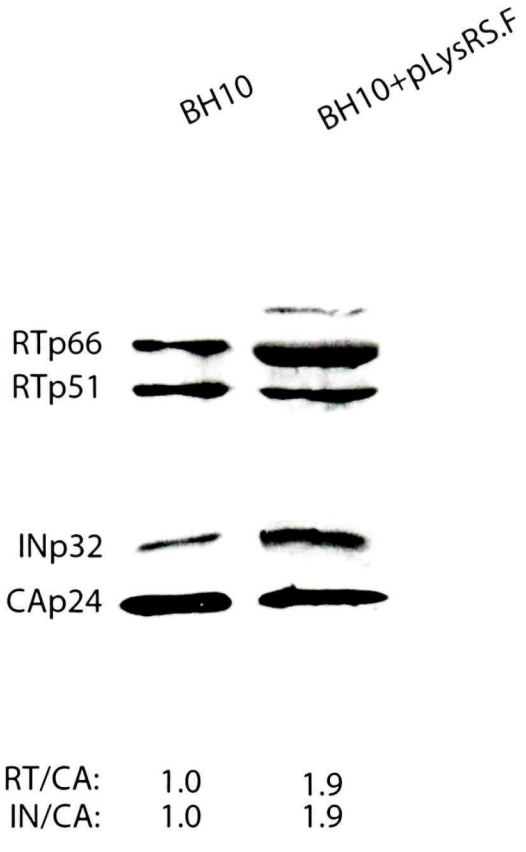

BH10
BH10Lys3
LysRS
CAp24
LysRS/CA:
1.0
2.6

Figure 3.4. Content of reverse transcriptase and integrase in HIV-1 containing increased amounts of LysRS.

Western blots were probed with anti-RT, anti-IN, and anti-CA. Bands were quantitated by phosphorimaging, and RT/CA and IN/CA ratios are listed below the lanes.


BH10
BH10+pLysRS.F
RTp66
RTp51
INp32
CAp24
RT/CA: 1.0 1.9
IN/CA: 1.0 1.9

Since, in a protease-negative virion, overexpression of LysRS results in close to a 2 fold increase in the viral content of LysRS and both $tRNA^{Lys}$ isoacceptors, without changing the incorporation of GagPol (98), increased GagPol incorporation into virions is not a likely explanation for the increased viral content of RT and IN. A more likely explanation is that the increased concentration of viral RT and IN probably reflects an increased resistance of GagPol to premature processing by viral protease, possibly through being part of the $tRNA^{Lys}$ packaging complex.

3.6. Discussion

In this work, we show that overexpression of either $tRNA^{Lys}$ isoacceptor or of LysRS results in a 2 to 3 fold increase in the RT/CA and IN/CA ratios in HIV-1. We interpret this to mean there is an increase in RT and IN in the virus, i.e., that CA/virion is not decreasing 2-3 fold. The increase in RT/CA and IN/CA could reflect an inhibition of premature proteolysis of GagPol before the completion of viral budding, thereby ensuring the complete encapsidation of the processing products. Increase in the RT/CA and IN/CA ratios occurs when either $tRNA^{Lys}$ isoacceptor or LysRS is overexpressed. Overexpression of either $tRNA^{Lys}$ isoacceptor does not result in an increase in either GagPol or total $tRNA^{Lys}$ incorporation into the viruses, but LysRS incorporation is increased. Overexpression of LysRS results in an increase in total $tRNA^{Lys}$ and LysRS incorporation, but again GagPol packaging remains unchanged. These results indicate 1) that LysRS may be responsible for making GagPol more resistant to proteolysis, and 2) the GagPol is not limiting for $tRNA^{Lys}$ packaging. Since LysRS incorporation into virions only requires Gag for incorporation (35), the LysRS may not interact directly with GagPol, but might nevertheless stabilize it by contribution to the overall conformational stability of a Gag/GagPol/$tRNA^{Lys}$/LysRS packaging complex.

Overexpression of LysRS results in increased incorporation of both LysRS and $tRNA^{Lys}$ into the virion. This could reflect a direct limitation of $tRNA^{Lys}$ packaging by the amount of LysRS available for forming a Gag/GagPol/LysRS/$tRNA^{Lys}$ packaging complex. However, the synthesis of new

LysRS might also increase the synthesis of new $tRNA^{Lys}$, and it may be the increased amount of $tRNA^{Lys}$ synthesis rather than LysRS synthesis that influences the amount of $tRNA^{Lys}$ packaged. It is also not clear why the increase in LysRS viral packaging resulting from overexpression of $tRNA^{Lys3}$ is not accompanied by an increase in total $tRNA^{Lys}$ packaging. An explanation for this will require further experimentation. LysRS can be packaged into Gag viral-like particles independently of $tRNA^{Lys}$ (35), and it is possible that overexpression of $tRNA^{Lys3}$ could stimulate new synthesis of LysRS without increasing total new $tRNA^{Lys}$ synthesis because of suppression of endogenous $tRNA^{Lys}$ synthesis.

CHAPTER 4

IDENTIFICATION OF $tRNA^{Asn}$ AND ASPARAGINYL SYNTHETASE IN HIV-1

This chapter was adapted from a manuscript prepared for submission to the Journal of Virology.

4.1. Preface

LysRS, like $tRNA^{Lys}$, is selectively packaged into HIV-1. Data presented in Chapter 2 has demonstrated that the limiting factor in the viral packaging of $tRNA^{Lys}$ is LysRS, and not GagPol, providing evidence indicating that the regulation of $tRNA^{Lys3}$ packaging involves interaction between itself and LysRS, and viral protein(s), for viral incorporation, and this likely occurs as part of a $tRNA^{Lys}$/LysRS/Gag/GagPol packaging complex.

In this next chapter, evidence will be presented to show that another tRNA synthetase, AsnRS, is selectively packaged into HIV-1. AsnRS and LysRS share structural similarities, and therefore it is not unlikely that AsnRS can be mistaken for LysRS by the viral packaging machinery. Interestingly, a non-$tRNA^{Lys}$ species, whose identity is most likely $tRNA^{Asn}$, is also selectively packaged into HIV-1. To date, there are no published descriptions of structural or biochemical similarities between $tRNA^{Lys}$ and $tRNA^{Asn}$ that are exclusive between the two. Data presented in this chapter will strongly support the hypothesis that the regulation of tRNA packaging in HIV-1 involves the tRNA's cognate tRNA synthatase.

4.2 Abstract

During HIV-1 assembly, the major $tRNA^{Lys}$ isoacceptors and the class IIB aminoacyl tRNA synthetase (aaRS) lysyl-tRNA synthetase (LysRS) are selectively packaged into the virion. We have identified one other packaged class IIB aaRS, asparagine tRNA synthetase (AsnRS), and we have tentatively identified another non- $tRNA^{Lys}$ species (identified as such by its partial T1 RNase digestion pattern) as $tRNA^{Asn}$, through its ability to hybridize with a DNA probe specific for $tRNA^{Asn}$. Since the incorporation of $tRNA^{Lys}$ into HIV-1 involves an interaction of a Gag/GagPol complex with a $tRNA^{Lys}$ / LysRS complex, and since AsnRS is structurally similar to LysRS, a similar mechanism may be involved in the incorporation of $tRNA^{Asn}$ into virions, i.e., the Gag/GagPol complex may be able to recognize the $tRNA^{Asn}$ /AsnRS complex.

4.3 Introduction

Cellular tRNA's are used in retroviruses as primers for reverse transcription. The tRNA is selectively packaged into the virion, where it is placed onto the viral genome at the primer binding site (PBS), and is used to prime the synthesis of minus (-) strand proviral cDNA. In avian sarcoma viruses (ASV) and avian leukosis viruses (ALV), $tRNA^{Trp}$ is the primer tRNA (203), (224), (265), (266). The tRNA primer for Moloney murine leukemia virus (Mo-MuLV) is $tRNA^{Pro}$ (109), (251). In HIV-1, the three $tRNA^{Lys}$ isoacceptors that are present in mammalian cells ($tRNA^{Lys1,2}$ and $tRNA^{Lys3}$ (207)) are all selectively packaged into the virus, but only $tRNA^{Lys3}$ is used as the primer for reverse transcription (126), (136), (162).

Selective packaging refers to an increase in concentration of a particular tRNA to total tRNA when going from the cytoplasm to the virus. For example, in HIV-1, the relative concentration of $tRNA^{Lys}$ in the cytoplasm of a mammalian cell is 5-6% of total tRNA, whereas in the virus, 50-60% of viral low molecular weight RNA is $tRNA^{Lys}$ [Mak, 1994 #1202]. In AMV, primer $tRNA^{Trp}$ makes up about 32% of the viral low molecular weight RNA, while in the cytoplasm, only 1.4% of tRNA is $tRNA^{Trp}$ (265). In some retroviruses, the primer tRNA is enriched to a lesser degree, particularly in AKR-MuLV, where 5-6% of cytoplasmic tRNA is $tRNA^{Pro}$, and in the virus the concentration is only 12-24% of viral low molecular weight RNA (265).

Of interest are the signals on $tRNA^{Lys}$ that target it for viral incorporation. Viral proteins that mediate $tRNA^{Lys}$ packaging are GagPol (148), (176) and Gag

(125). *In vivo* studies have shown that particles composed of Gag alone do not selectively package $tRNA^{Lys}$, but viral particles composed of both Gag and GagPol do selectively incorporate $tRNA^{Lys}$ (176). Recently, we found that a $tRNA^{Lys}$ binding protein, lysyl-tRNA synthetase (LysRS), the protein that aminoacylates $tRNA^{Lys}$, is also selectively packaged into HIV-1 (35). Gag alone is sufficient for incorporation of LysRS into Gag VLPs [Cen, 2001 #277], although GagPol is required for $tRNA^{Lys}$ incorporation as well (176). We have also shown that LysRS is a limiting factor for $tRNA^{Lys}$ packaging, i.e., increasing LysRS expression results in more $tRNA^{Lys}$/virion (98). Furthermore, the ability of wild-type or mutant $tRNA^{Lys3}$ to be aminoacylated *in vivo* is directly correlated with its ability to be packaged into HIV-1 (135). Based upon these findings, we have postulated a model for the $tRNA^{Lys}$ packaging complex, in which the Gag/GagPol complex interacts with the $tRNA^{Lys}$/LysRS complex. We hypothesize that Gag interacts with LysRS, and GagPol interacts with $tRNA^{Lys}$, and that this complex is carried into the assembling virion.

Analysis of the low molecular weight RNA in HIV-1 by two dimensional polyacrylamide gel electrophoresis (2D PAGE) reveals 3 or 4 major spots. Three of these spots have been identified as $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ (136), but the fourth spot, which in different viral preparations can vary in intensity, relative to the $tRNA^{Lys}$ species, has not been identified. In this paper, we have tentatively identified this species as $tRNA^{Asn}$, and show that that the enzyme which aminoacylates $tRNA^{Asn}$, asparagine tRNA synthetase (AsnRS) is also found in the virus.

4.4. Materials and methods

4.4.1 Plasmid construction.

SVC21.BH10 is a simian virus 40-based vector containing wild-type HIV-1 proviral DNA, constructed as previously described (123). Plasmid pM368 contains cDNA encoding full-length (1-597 amino acids) human LysRS, as previously described (233). The cDNA was PCR-amplified, and digested with EcoR1 and Xho1, whose sites were placed in each of the PCR primers. For expression in COS7 cells, the PCR DNA fragments were cloned into pcDNA 3.1 (Invitrogen) to obtain pLysRS.F, expressing full length LysRS.

4.4.2 Virus infection/transfection and purification.

COS7 cells were transfected using the calcium phosphate method as previously described (176). Supernatant was collected 63 hours post-transfection. Viruses were pelleted from culture medium by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 1 hour. The viral pellets were then purified by centrifugation in a Beckman SW41 rotor at 26,500 rpm for 1 hour through 15% sucrose onto a 65% sucrose cushion. The band of purified virus was removed and pelleted in 1X TNE in a Beckman Ti45 rotor at 40,000 rpm for 1 hour. Viral genomic RNA was extracted using guanidium isothiocynate, as previously described (136). The RNA pellets were dissolved in 5mM Tris-HCl, aliquoted and stored at -70°C.

4.4.3. ^{32}pCp labeling of tRNA

The RNA was 3′- labeled with ^{32}pCp and T4 RNA ligase as previously described (28), (137). ^{32}pCp was made as follows: 5 mCi of gamma-^{32}P-ATP (specific activity 3000 Ci/mmole, Dupont, Canada) was dried down in a microcentrifuge tube using N_2. 100 ul of the following reaction solution was added (reaction solution: 50 mM Tris-HC1, pH 9.2, 5 mM $MgCl_2$, 3 mM dithiothreitol, 5% bovine serum albumin, 1 uM 3'-cytidine monophosphate (3'-CMP), and 10 units T4 kinase). The reaction was incubated at 37^{0}C for 3 hours. After labeling of the RNA with ^{32}pCp, free ^{32}pCp was removed from the labeled macromolecules either using G-50 Sephadex (Pharmacia) home-made spin columns, equilibrated with TE buffers (10 mM Tris, 7.5; 1 mM EDTA), or during the electrophoresis run. Before analysis by polyacrylamide electrophoresis, the samples were heated at 90°C for 2 minutes.

2D PAGE electrophoresis of labeled viral RNA was carried out at 4°C using the Hoefer SE620 gel electrophoresis apparatus. Gel size was 14 cm x 32 cm. The first dimension was run in a 10% polyacrylamide/7 M urea gel for approximately 16 hours at 800 volts, until the bromphenol blue dye was beginning to elute from the bottom of the gel. After autoradiography, the piece of gel containing RNA was cut out and embedded in a second gel (20% polyacrylamide/7 M urea) and run for 30 hours (25 watt limiting), followed by autoradiography. All electrophoretic runs were carried out in 0.5 x TBE (1 X TBE is 50 mM Tris, 5 mM boric acid, 1 mM EDTA-Na_2).

4.4.4. 1D and 2D PAGE.

Electrophoresis of ^{32}pCp-labelled viral RNA was carried out at 4°C with the Hoeffer SE620 gel electrophoresis apparatus. The gel size was 14 by 32 cm. The first dimension was run in an 11% polyacrylamide-7M urea gel for 16 hours at 800 V. After autoradiography, the piece of gel containing RNA was cut out, and run for 30 hours (25 Watt limiting); this was followed by autoradiography. All electrophoretic runs were carried out in 0.5X TBE. The electrophoretic gel patterns shown in this paper show only low molecular weight RNA, since the high-molecular weight viral genomic RNA cannot enter into the polyacrylamide gels. Furthermore, these patterns represent only the most abundant tRNA species present, since longer film exposures will reveal the presence of more minor-abundance species.

4.4.5. Northern blotting and hybridization.

Transfer of labeled ^{32}pCp-labelled RNA to Hybond N+ nylon membrane (Amersham) took place in 1X TBE (50 mM Tris, 5mM boric acid, 1mM EDTA-Na_2) for 1 hour at 100 volts. The blot was then left sealed in an airtight plastic bag for approximately two months for the purpose of allowing the ^{32}pCp-labelled RNA to decay. After confirming that there was no detectable radiolabel left on the membrane (by autoradiography), the blot was probed with a 5′ ^{32}P-end-labelled 18-mer DNA probe specific for the 3′ end of $tRNA^{Asn}$ (5'-TGGCGTCCCTGGGTGGGC-3'). Following autoradiography of the hybridized probe, the membrane was stripped according to the manufacturer's instructions,

and was re-probed with a 5′ ^{32}P-end-labelled 18-mer DNA probe specific for the 3′ end of $tRNA^{Lys3}$ (5′-TGGCGCCCGAACAGGGAC-3′).

4.4.6. RNase T1 digests

^{32}pCp-labelled RNA was run on 2D PAGE, and labeled RNA species were excised from the gel, and eluted overnight in elution buffer (0.5M ammonium acetate, 1mM EDTA, 0.2% SDS) at 37°C. The samples were then purified with phenol chloroform and ethanol precipitated. RNase T1 footprints were obtained by partial enzymatic hydrolysis, by incubating about 10^5 cpm of 3' end labeled RNA sample for 30 minutes at 55°C in digestion buffer (16 mM NaAc, pH 5.0; 0.8 mM EDTA; 0.02% xylene cyanole FF (XC); 0.04% bromophenol blue; 1 ug of *E. coli* carrier tRNA; 3.5 M urea) with 0.2 U/ul of RNase T1, which specifically hydrolyzes RNA at G residues. The reaction was stopped by cooling to 0°C and all products were analyzed by 1D PAGE.

4.4.7. Protein Analysis.

Viral particles were purified as described above, and viral proteins were extracted with RIPA buffer (10 mM Tris, pH 7.4, 100mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP40, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin A, 100 mg/ml PMSF). The viral lysates were analyzed by SDS PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amersham Pharmacia). Detection of protein by Western blotting utilized a polyclonal antibody that is specifically reactive with human lysyl tRNA synthetase

(a kind gift from Kiyotaka Shiba, Japan), and human anti-sera that is specifically reactive for asparaginyl tRNA synthetase. Detection of HIV proteins was performed by enhanced chemiluminescence (NEN Life Sciences Products) using as secondary antibodies anti-rabbit (lysyl tRNA synthetase), or anti-human (asparaginyl tRNA synthetase) both obtained from Amersham Life Sciences. Western blot stripping was done by washing the blot 4 times for 5 minutes in PBS-Tween (0.5% Tween). The blot was then incubated for 30 minutes at 50°C in stripping buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 100mM 2-mercaptoethanol). Following this incubation, the blot was washed 6 times for 5 minutes in PBS-Tween, then blocked again and re-probed with a different anibody.

4.4.8. Purification of LysRS

To purify wild-type LysRS, 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. The protein was overexpressed in *Escherichia coli* and purified as previously described (233).

4.5. Results

4.5.1 Asparaginyl-tRNA synthetase can be selectively packaged into HIV-1.

Selective packaging of LysRS means that other aaRSs investigated were not found in HIV-1. We have thus far reported that neither prolyl-tRNA synthetase and isoleucyl-tRNA synthetase (35), nor tryptophanyl-tRNA synthetase (33), are found in HIV-1. However, in this report, we have obtained evidence that AsnRS is packaged into HIV-1. Protein from purified HIV-1 or cell protein was resolved by 1D SDS PAGE, transferred to nitrocellulose, and probed with human anti-sera specific for AsnRS. The predicted molecular weight of human AsnRS is 62,902 Da [Shiba , 1998 #2425], and Figure 4.1 shows the hybridization of anti-AsnRS in both cell and viral lysates with AsnRS. Both cell and virus samples contain two different sized bands, one apparently full length at M_r = 63,000 Da, the other apparently truncated at M_r= 55,000 Da. Truncation of tRNA synthetases is not uncommon; truncation of LysRS is also seen in HIV-1 (35).

Full length LysRS has a M_r = 68,000 Da, which is larger, but similar, to the M_r of AsnRS. Figure 4.2 shows the specificity of the anti-AsnRS antibody for its target, AsnRS. The Western blot, showing viral lysate (lane 1) and purified C-terminal His-tagged LysRS protein (lanes 2, 3 and 4, representing 2, 5, and 10 ng respectively), was probed first with anti-AsnRS (panel A), stripped, then re-probed with anti-LysRS (panel B). The data in Panel B indicates that there is between 2 and 5 ng of LysRS present in the HIV-1 sample, and yet anti-AsnRS

Figure 4.1. Incorporation of AsnRS into HIV-1.

Western blot of COS7 cell lysate (cell) and viral lysate (virus). Viral lysates were produced from virus collected from COS7 cells transfected with SVC21.BH10 (a simian virus 40-based DNA vector containing wild-type HIV-1 proviral DNA). Blots were probed with anti-AsnRS.

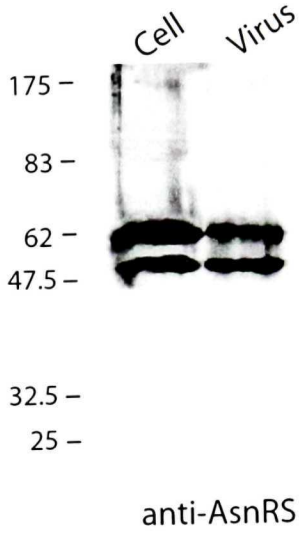
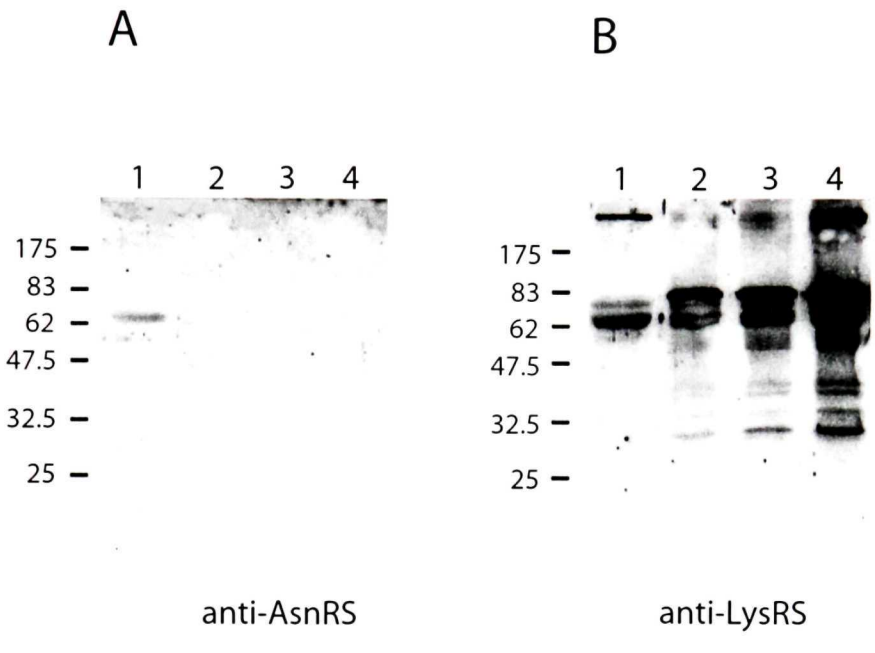

Cell
Virus
175 –
83 –
62 –
47.5 –
32.5 –
25 –
anti-AsnRS

Figure 4.2. Specificity of anti-AsnRS.

Western blot of viral lysate (lane1) and purified C-terminal His-tagged LysRS protein (lanes 2, 3, and 4, representing 2,5, and 10 ng, respectively).

A. Hybridization of anti-AsnRS with AsnRS.

B. Hybridization of anti-LysRS with LysRS.


A
B
1
2
3
4
175
83
62
47.5
32.5
25
anti-AsnRS
anti-LysRS

cannot detect up to the 10 ng of LysRS detected, i.e., there is no detectable cross-reactivity of anti-AsnRS with LysRS. Therefore, the viral species detected in lane 1 in panel A is AsnRS, and not LysRS.

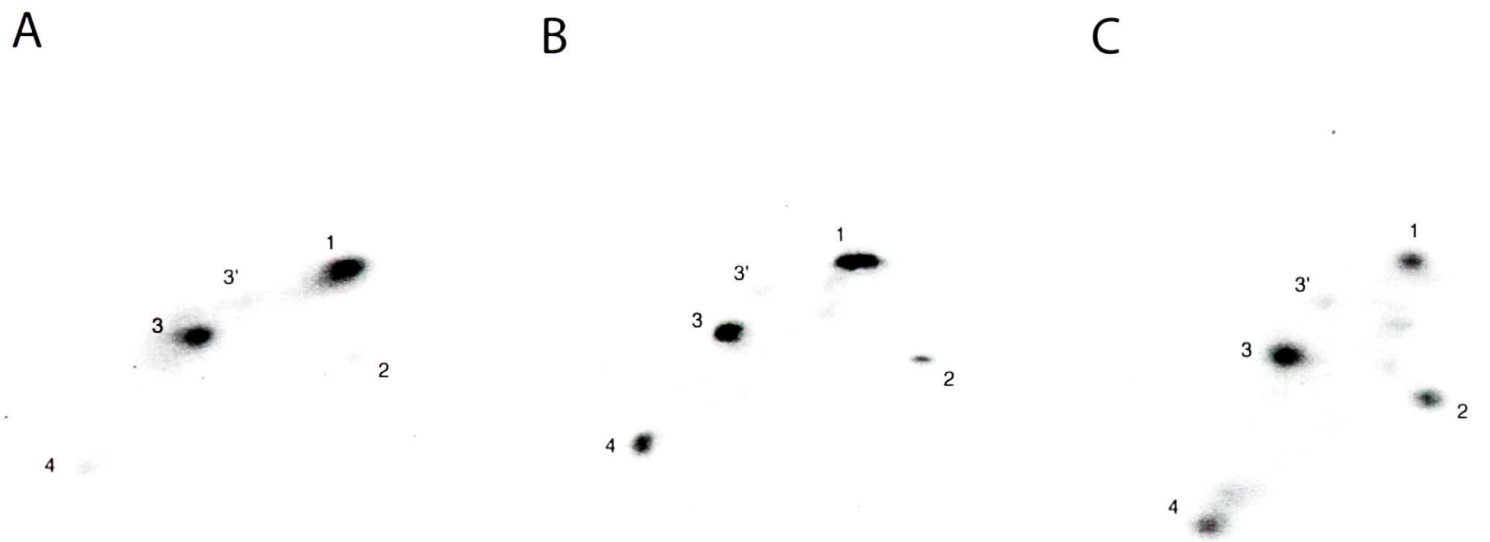
LysRS may serve as the target by which Gag/GagPol interacts with $tRNA^{Lys}$ /LysRS so as to incorporate both $tRNA^{Lys}$ and LysRS into HIV-1. AsnRS and LysRS are structurally similar, i.e., they are both, along with aspartyl-tRNA synthetase, Class 2B synthetases. It is therefore possible that the Gag/GagPol complex may also be able to recognize to some degree the $tRNA^{Asn}$/AsnRS complex found in the cell. We therefore next examined whether $tRNA^{Asn}$ is found in the virion.

4.5.2 $tRNA^{Asn}$ is sometimes selectively packaged into HIV-1.

Figure 4.3, A-C, shows the 2D PAGE pattern of HIV-1 low molecular weight RNA. Total viral RNA was purified from HIV-1 produced in transfected COS7 cells, and was 3´- end labeled with ^{32}pCp. The samples were resolved by 2D PAGE,and the labeled RNA was transferred to a nylon membrane and exposed to film. Spots 1 and 2 on the 2D PAGE have previously been identified as $tRNA^{Lys1,2}$, and spot 3 has been identified as $tRNA^{Lys3}$ (136). Spot 3' has been identified in this report as a $tRNA^{Lys3}$ species (see figure 4.4). There is also a spot 4, whose intensity, relative to the $tRNA^{Lys}$ spots, varies with different viral preparations, as can be seen in the three viral preparations represented in panels A-C. The spots from panel B were excised, and exposed to partial

Figure 4.3. 2D PAGE of HIV-1 RNA

Total viral RNA was extracted from 3 different viral preparations (seen in panels A, B, and C), was 3' end labeled with ^{32}pCp-labelled, and was analyzed by 2D PAGE. Spots 1,2 and 3 have been presented previously (136), and are identified as: 1, $tRNA^{Lys1,2}$; 2, $tRNA^{Lys1,2}$; 3, $tRNA^{Lys3}$. Spot 3' is identified herein as $tRNA^{Lys3}$ (see Figure 4.4) and spot 4 is tentatively identified herein as $tRNA^{Asn}$ (see Figure 4.4 and 4.5). Note the variability in the viral concentration of spot 4.


A
1
3'
3
2
4
B
1
3'
3
2
4
C
1
3'
3
2
4

digestion by RNase T1, as described in Materials and methods. The fragments labeled at the 3′ terminus were resolved by 1D PAGE, and the patterns are shown in Figure 4.4. We have previously reported the T1 RNase digestion pattern of purified human placental $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ (136), and the digestions patterns shown for spots 2 and 3 match with these patterns. In addition, the T1 RNase digestion pattern of spot 3′ indicates that it is an isoform of $tRNA^{Lys3}$. However, the T1 RNase digestion pattern of spot 4 does not match with any of the $tRNA^{Lys}$ species, indicating that it is not a $tRNA^{Lys}$ isoacceptor.

To see whether a DNA probe specific for $tRNA^{Asn}$ would specifically hybridize to the RNA in spot 4, the labeled tRNA spots from another gel were transferred from the gel to a nylon membrane (shown in Figure 4.5A), which was then sealed in plastic and incubated at room temperature for 2 months to allow the decay of radiolabelled RNA to remove its signal. It was then probed with a 5' radiolabelled oligonucleotide complementary to the 3' end of $tRNA^{Asn}$, and hybridization of the probe to spot 4, as shown in Figure 4.5B, indicates that this RNA species is most likely $tRNA^{Asn}$. As a control, Figure 4.5C shows the same blot, which was stripped and re-probed with a 5' radiolabelled oligonucleotide complementary to the 3' end of $tRNA^{Lys3}$, which hybridized to spots 3 and 3'.

Figure 4.4. Identification of $tRNA^{Lys}$ isoacceptors by partial T1 RNase digestion.

Partial T1 RNase digests of the spots excised from the gel in Figure 4.3B are analyzed using 1D PAGE to separate the RNA fragments. The T1 RNase digestion patterns for purified human placental $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$, as well as for spots 3, 2, and 1 in HIV-1 RNA, have been presented previously (136). Lane numbers refer to spot numbers, as in Figure 4.3. Spots: 1, $tRNA^{Lys1,2}$; 2, $tRNA^{Lys1,2}$; 3, $tRNA^{Lys3}$; 3′, $tRNA^{Lys3}$; 4, non-$tRNA^{Lys}$.

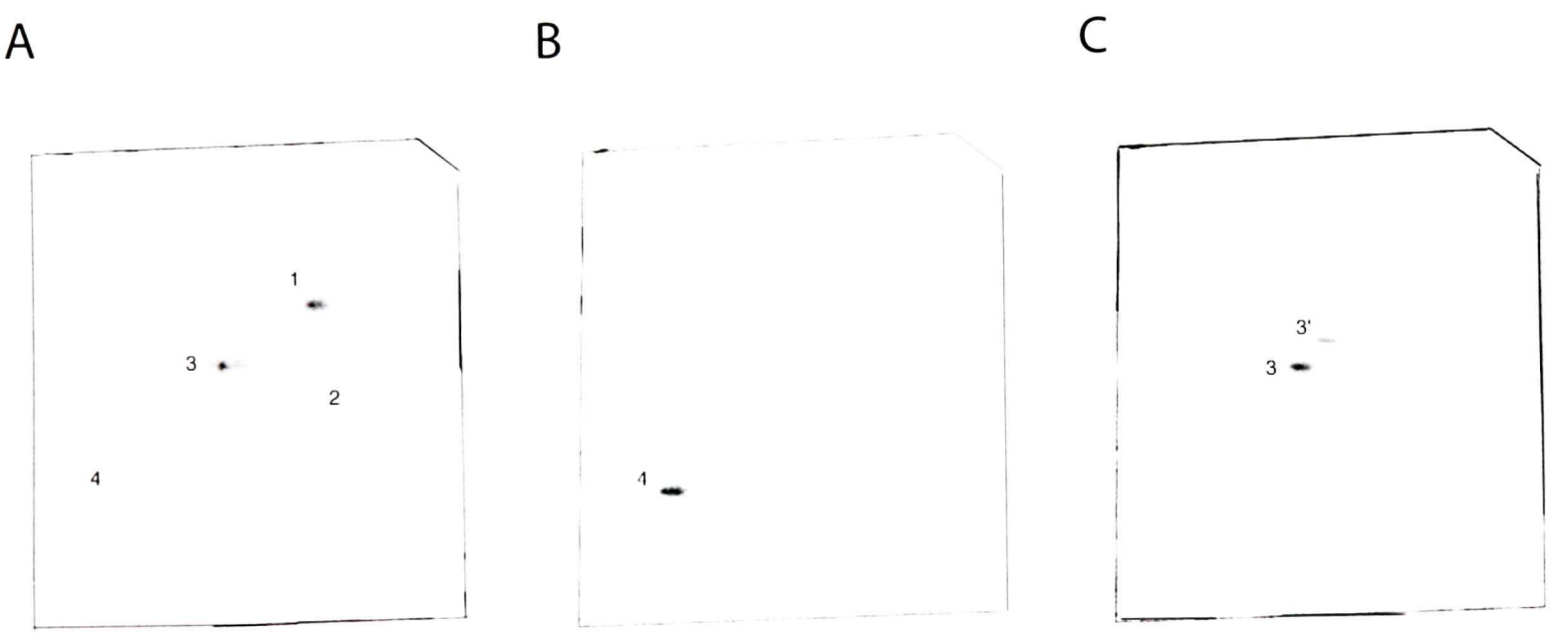

3
3'
1
2
4

Figure 4.5. Northern blotting of 2D PAGE gel and identification of tRNA species by hybridization.

A. Northern blot of 2D PAGE gel of total viral RNA from transfected COS7 cells. Spot numbers are identified as in Figure 4.3.

B. Northern blot from Figure 4.5A, after 2 months of radiolabel decay. The blot was probed with a 5' end-labelled DNA oligonucleotide specific for $tRNA^{Asn}$ which was hybridized to spot 4.

C. Northern blot from Figure 4.5B. The blot was stripped and re-probed with a 5' end-labeled oligonucleotide specific for $tRNA^{Lys3}$, which was hybridized to spot number 3 (previously identified as $tRNA^{Lys3}$), and to spot number 3' (also identified herein as a $tRNA^{Lys3}$ isoform).


A
1
3
2
4
B
4
C
3'
3

4.6 Discussion

The results presented here indicate that $tRNA^{Asn}$ and AsnRS can be selectively packaged into HIV-1. While the function of $tRNA^{Lys3}$ as a primer for reverse transcription is well known, there is no known function for $tRNA^{Asn}$ or AsnRS in the virus. AsnRS and LysRS are both members, along with aspartyl tRNA synthetase, of a structurally similar class of aminoacyl tRNA synthetases termed Class 2B (83) (84). These enzymes are found as homodimers with an N-terminal anticodon-binding domain and with a topology known as an oligonucleotide-binding (OB) fold. Because of their structural similarity, we postulate that the Gag/GagPol complex, which normally interacts with the $tRNA^{Lys}$ /LysRS complex, may occasionally bind to AsnRS instead of LysRS, and carry both AsnRS and $tRNA^{Asn}$ into the virion. What is behind the variability of $tRNA^{Asn}$ incorporation is not known.

CHAPTER 5

GENERAL DISCUSSION

5.1. The importance of the select packaging of $tRNA^{Lys3}$ in HIV-1

$tRNA^{Lys3}$ is the primer for reverse transcriptase in HIV-1, and its 3' 18 terminal nucleotides are complementary to the PBS on the viral genomic RNA. In Chapter 2, we showed that the more $tRNA^{Lys3}$ that was packaged into the virus, the more $tRNA^{Lys3}$-initiated reverse transcription occurred, and the more infectious the viral population became. The amount of $tRNA^{Lys3}$/virion required for maximum occupancy of the primer binding site in a virion is unknown, but we have interpreted our data as indicating that when more cytoplasmic $tRNA^{Lys3}$ is available, a greater number of virions in the population have the opportunity to package that amount of $tRNA^{Lys3}$ required for maximum initiation of reverse transcription.

Attempts have been made to construct mutant viruses that will stably use other tRNA's as RT primers by changing the PBS to be complementary to other tRNA's (68), (170), (262). In most cases, the altered priming binding site reverts back to wild-type $tRNA^{Lys3}$. However, in one case, the PBS was made complementary to the 3′ termini of $tRNA^{His}$, plus an A-rich loop just upstream of the PBS, which is believed to interact with the anticodon of $tRNA^{Lys3}$, was made complementary to the anticodon loop of $tRNA^{His}$. This mutant virion, termed His-AC-GAC, was found to stably prime reverse transcription using $tRNA^{His}$ (260). Although this mutant virus did not appear to specifically package $tRNA^{His}$, it maintained a $tRNA^{His}$ PBS over many viral generations, and was able to replicate, albeit with lower replication kinetics than wild type HIV-1. Thus, when forced to do so, HIV-1 can find ways to initiate reverse transcription with an

alternate tRNA primer present in viral concentrations much lower than $tRNA^{Lys}$. It is not known whether other mutations in the viral genome were also required for this phenomenon to occur.

Nevertheless, under normal conditions, it appears that HIV-1 utilizes selective packaging of primer $tRNA^{Lys3}$ as a means to achieve optimum growth, since higher replication rates would be expected to have a more positive effect upon the successful establishment of infection in the host. If the His-AC-GAC mutant was to attempt to establish a productive infection in a host, it is possible that such an attempt would not be as successful as it would be for the wild-type virus because of the mutant virus' lower replication kinetics, which might allow time for the host to mount a stronger immune response.

5.2. Regulation of packaging of $tRNA^{Lys3}$ in HIV-1

The selective packaging of $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ into HIV-1 during viral assembly requires the participation of GagPol (176). While work in our laboratory has indicated that the thumb region of reverse transcriptase in GagPol contains an important binding site for $tRNA^{Lys}$ which is utilized during $tRNA^{Lys}$ packaging into virions (148), the signal on $tRNA^{Lys}$ that is recognized by viral proteins has not been known. However, recent work in our laboratory has indicated that LysRS may be that signal. LysRS is selectively packaged into HIV-1 (35), and the interaction between $tRNA^{Lys}$ and LysRS is required for $tRNA^{Lys}$ to be packaged into HIV-1 (135).

In Chapter 2 (98), we have provided further evidence that LysRS may limit the amount of $tRNA^{Lys}$ incorporated into HIV-1 by showing that overexpression of LysRS results in up to a two-fold increase in the viral incorporation of both $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$, without changing the incorporation of GagPol. This suggests that the limiting factor in $tRNA^{Lys}$ packaging into HIV-1 is not GagPol, but LysRS. Of course, the possibility exists that since overexpression of LysRS results in an increased production of cytoplasmic $tRNA^{Lys}$, it may be the increase in cytoplasmic $tRNA^{Lys}$ rather than a direct interaction with LysRS that is responsible for the increase in $tRNA^{Lys}$ packaging into virions. However, recent unpublished work in our laboratory suggests that this is not so; Dr. Shan Cen has shown that overexpression of a mutant, N-terminally truncated LysRS that is incapable of binding to $tRNA^{Lys}$ results in the overexpression of cytoplasmic $tRNA^{Lys}$, but no increase in $tRNA^{Lys}$ incorporation into viruses is seen even though viral LysRS content increases as a result of the incorporation of both endogenous wild type and mutant LysRS. This indicates that even with increased cytoplasmic $tRNA^{Lys}$, LysRS itself must be able to bind to the $tRNA^{Lys}$ to carry it into the virion.

More surprising is the fact that overexpression of $tRNA^{Lys3}$ results in a greater incorporation of LysRS (Chapter 3). Data presented in Chapter 2 (98), as well as previous work in our laboratory (126), shows that the cytoplasmic overexpression of $tRNA^{Lys3}$ results in an increase in viral $tRNA^{Lys3}$ with a corresponding decrease of $tRNA^{Lys1,2}$, which results in the total viral $tRNA^{Lys}$

remaining constant. Why, then, does incorporation of LysRS into virions increase, if total $tRNA^{Lys}$ incorporation does not? This remains unexplained.

5.3. Stabilization of GagPol by tRNALys or LysRS.

Data presented in Chapter 3 shows that the overexpression of either $tRNA^{Lys3}$, $tRNA^{Lys1,2}$, or LysRS results in an increase the content of RT and IN in the viral population. Since GagPol incorporation into HIV-1 is not altered under these conditions (Chapter 2 (98)), this data may indicate that GagPol is protected from premature viral protease processing while it is in the $tRNA^{Lys}$ packaging complex, i.e., precursor protein processing is delayed until the immature virion is completely encapsidated with membrane, so as not to lose smaller processed products back into the cell cytoplasm.

5.4. Incorporation of $tRNA^{Asn}$ and AsnRS into HIV-1.

Recent work in our laboratory has examined the incorporation of three aminoacyl-tRNA synthetases (LysRS, tryptophanyl-tRNA synthetase (TrpRS), and prolyl-tRNA synthetase (ProRS) into either HIV-1, Rous sarcoma virus (RSV), or murine leukemia virus (MuLV). RSV using $tRNA^{Trp}$ as a primer, and MuLV uses $tRNA^{Pro}$ as a primer. We have found that, of these three aminoacyl-tRNA synthetases examined, HIV-1 contains only LysRS, RSV contains only TrpRS, and MuLV contains none of these three aminoacyl-tRNA synthetases (33). This work indicates a tight regulation in HIV-1 and RSV between primer tRNA incorporation and its cognate aminoacyl-tRNA synthetase.

Nevertheless, in the process of investigating what other aminoacyl-tRNA synthetases are incorporated into HIV-1, we have only found AsnRS (Chapter 4) and LysRS (35) thus far. In Chapter 4, we also provided evidence using 2D PAGE that the only major tRNA isoacceptor incorporated into HIV-1 other than the $tRNA^{Lys}$ isoacceptors is $tRNA^{Asn}$. AsnRS is classified in the same aminoacyl-tRNA synthetase structural class as LysRS (Class 2B synthetases), which suggests that AsnRS may be erroneously recognized by the viral protein(s) that would target LysRS for incorporation, and as a result of AsnRS's packaging, its cognate tRNA, $tRNA^{Asn}$, is also packaged. This hypothesis implies that GagPol can also recognize $tRNA^{Asn}$. It is not known how discriminatory GagPol binding is to different tRNAs, compared to the discriminatory binding of Gag to different aminoacyl-tRNA synthetases.

The data in this thesis has demonstrated the importance of the select packaging of primer $tRNA^{Lys3}$ to maximize the ability of HIV-1 to replicate through the optimization of the virus' ability to initiate reverse transcription from the primer tRNA. It has also given further credence to the role of LysRS in regulating $tRNA^{Lys}$ incorporation into virions, and has indicated the importance of LysRS in increasing viral infectivity through its ability to increase the viral concentrations of GagPol cleavage products, by preventing premature proteolysis of GagPol during packaging. Interactions occurring in the $tRNA^{Lys}$ packaging complex, such as those between Gag/LysRS or GagPol/$tRNA^{Lys}$, represent a new set of targets for anti-HIV-1 drugs to be used in the fight against AIDS.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The following is a summary list of my original contributions from the thesis to the research community under the supervision of Dr. Lawrence Kleiman.

1) Increasing the viral $tRNA^{Lys3}$ concentration is accompanied by an increase in $tRNA^{Lys3}$ annealing to the PBS, and an increase in viral infectivity.

2) Overexpression of viral LysRS results in an almost doubling of viral $tRNA^{Lys}$ concentration in protease negative HIV-1.

3) LysRS is a limiting factor in $tRNA^{Lys}$ packaging, and GagPol is not.

4) GagPol is stabilized by $tRNA^{Lys}$ isoacceptors and LysRS.

5) AsnRS and $tRNA^{Asn}$ are selectively packaged into HIV-1.

6) The select packaging of a tRNA into HIV-1 appears to be facilitated by the select packaging of its cognate synthetase.

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