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The Packaging and Annealing of Primer tRNA^{Lys3} in HIV-1

By

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A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

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To my Parents

,

Jamal and Sabihah Saadatmand,

without whom I would have never been the person that I am today; for all your unconditional love, sacrifices, patience, and encouragement.

"No words to express my gratitude for you"

And

To my mentor,

Dr. Lawrence Kleiman,

the person who believed in me all along, for your guidance, patience, friendship and encouragement.

"We did it"

September 2008

Abstract

Reverse transcription in HIV-1 (human immunodeficiency virus type 1) is initiated from a tRNA, $tRNA^{Lys3}$, that is annealed to the primer binding site (PBS) in the 5′ region of viral RNA. This tRNA, along with the other major $tRNA^{Lys}$ isoacceptors, $tRNA^{Lys1,2}$, is selectively packaged into HIV-1 during its assembly. The formation of a $tRNA^{Lys}$ packaging/annealing complex is believed to involve the interaction between a Gag/GagPol/viral complex with a lysyl-tRNA synthetase (LysRS)/ $tRNA^{Lys}$ complex, with Gag interacting specifically with LysRS, and GagPol interacting with both Gag and $tRNA^{Lys}$. In fact, Gag particles alone will package LysRS, but GagPol, which binds $tRNA^{Lys}$, is also required for incorporation of the $tRNA^{Lys}$.

The model we propose for the $tRNA^{Lys}$ packaging/annealing complex predicts a possible interaction between LysRS and Pol sequences in GagPol, which might facilitate transfer of $tRNA^{Lys3}$ from LysRS to the reverse transcriptase (RT) thumb domain where $tRNA^{Lys3}$ binds. In this work, we demonstrate that, in addition to its interaction with Gag, LysRS also interacts with sequences within the connection/RNaseH domains in RT. Since these RT domains are not required for $tRNA^{Lys}$ packaging into HIV-1, the LysRS/Pol interaction is probably not involved in the transfer of $tRNA^{Lys3}$ to RT. The LysRS/Pol interaction may instead be involved in $tRNA^{Lys3}$ annealing since the connection domain in RT has been found to be required for this process. Also, since an interaction has been reported between Gag and Pol sequences in GagPol, we also investigated whether the Gag/LysRS/Pol interaction played an important role in stabilizing the Gag/Pol interaction, and found, using siRNA to LysRS, that it did not.

$tRNA^{Lys3}$ annealing to viral RNA is promoted by nucleocapsid sequences in Gag and by mature NCp7, and we have examined the roles of Gag and NCp7 in this process. Gag- and NC-facilitated $tRNA^{Lys3}$ annealing to HIV-1 RNA were measured both *in vivo* and *in vitro*, indirectly by the ability of annealed $tRNA^{Lys3}$ to prime reverse transcription, and directly by measuring the occupancy of the PBS by $tRNA^{Lys3}$. While $tRNA^{Lys3}$ annealing can be carried out by both Gag and NCp7, exposure (*in vivo* or *in vitro*) of the $tRNA^{Lys3}$/viral RNA complex to NCp7 is required for optimum placement of the $tRNA^{Lys3}$. This is indicated by 1) $tRNA^{Lys3}$'s reduced ability to incorporate the first dNTP, dCTP, and 2) its more ready displacement from the PBS by DNA synthesized from a downstream primer.

It has been previously demonstrated that APOBEC3G (A3G) can inhibit $tRNA^{Lys3}$ annealing to viral RNA, and we have used A3G to further dissect the roles of Gag and NCp7 in annealing, both *in vitro* and *in vivo*. Experiments studying how APOBEC3G (A3G) inhibits $tRNA^{Lys3}$ annealing indicate that in protease-positive viruses, Gag-facilitated $tRNA^{Lys3}$ annealing may only play a minor role. *In vivo* and *in vitro*, A3G only inhibits NCp7-facilitated annealing, and not Gag-facilitated annealing. Nevertheless, while Gag is able to show 70-80% of the annealing efficiency of NCp7 in a protease-negative virus, A3G can reduce annealing efficiency in protease-positive viruses to 40%. This appears to be due to the fact that, *in vitro*, the presence of NCp7 makes prior Gag-facilitated annealing susceptible to A3G. This suggests that in wild type viruses, any Gag-facilitated annealing of $tRNA^{Lys3}$ to viral RNA that does occur is altered through an A3G-susceptible re-annealing by NCp7.

Résumé

La transcription inverse du VIH-1 (virus de l'immunodéficience humaine de type 1) est amorcée avec l'ARN de transfert, $tRNA^{Lys3}$, qui se fixe au site de liaison d'amorce (PBS) dans la région 5′ de l'ARN viral. Avec d' autres isoaccepteurs majeurs $tRNA^{Lys1,2}$, $tRNA^{Lys3}$ est sélectivement encapsidé dans le VIH-1 pendant son assemblage. La formation du complexe encapsidation/fixation du $tRNA^{Lys}$ est censée impliquer l'interaction entre le complexe viral Gag/GagPol et le complexe de la synthétase lysyl-tRNA (LysRS)/ $tRNA^{Lys}$, avec Gag qui interagit spécifiquement avec LysRS, et GagPol interagissant aussi bien avec Gag et $tRNA^{Lys}$. En effet, seules les particules de Gag encapsident LysRS, alors que GagPol, qui lie $tRNA^{Lys}$, est également requis pour l'incorporation des $tRNA^{Lys}$.

Notre modèle pour le complexe encapsidation/fixation du $tRNA^{Lys}$ prévoit une interaction entre les séquences de LysRS et de Pol dans GagPol, ce qui pourrait faciliter le transfert de $tRNA^{Lys3}$ à partir du LysRS vers le domaine "pouce" de la réverse transcriptase (RT) où le $tRNA^{Lys3}$ se lie. Dans ce travail, nous avons montré qu'en plus de son interaction avec Gag, LysRS interagit également avec des séquences à l'intérieur domaine de connection/RNaseH de la RT. Puisque ces domaines de la RT ne sont pas requis pour l'encapsidation du $tRNA^{Lys}$ dans le VIH-1, l'interaction de LysRS/Pol n'est probablement pas impliquée dans le transfert de $tRNA^{Lys3}$ vers la RT. L'interaction de LysRS/Pol peut être plutôt impliquée dans la fixation du $tRNA^{Lys3}$ vu que domaine de connection de la RT a été trouvé nécessaire pour ce processus. En outre, puisqu'une interaction a été rapportée entre les séquences Gag et Pol dans GagPol, nous avons

également étudié si l'interaction de Gag/LysRS/Pol joue un rôle important dans la stabilisation de l'interaction de Gag/Pol, mais en utilisant des siRNA contre LysRS, ceci n'a pas été prouvé.

La fixation du $tRNA^{Lys3}$ à l'ARN viral est favorisée par des séquences de la nucléocapside dans Gag et par la NCp7 mature et nous avons examiné les rôles de Gag et NCp7 dans ce processus. La fixation de $tRNA^{Lys3}$ à l'ARN du VIH-1 facilitée par Gag et NC a été mesurée *in vivo* et *in vitro*, indirectement par la capacité du $tRNA^{Lys3}$ fixé d'amorcer la transcription inverse, et directement en mesurant l'occupation du PBS par le $tRNA^{Lys3}$. Tandis que la fixation du $tRNA^{Lys3}$ peut être effectué par Gag et NCp7, l'exposition (*in vivo* ou *in vitro*) du complexe $tRNA^{Lys3}$/ARN viral à la NCp7 est requise pour un placement optimum du $tRNA^{Lys3}$. Ceci est indiqué par 1) La capacité réduite du $tRNA^{Lys3}$ d'incorporer le premier dNTP, dCTP, et 2) son déplacement du PBS par l'ADN synthétisé d'une amorce en aval.

Il a été précédemment montré qu'APOBEC3G (A3G) peut inhiber la fixation du $tRNA^{Lys3}$ à l'ARN viral, et nous avons utilisé A3G, *in vitro* et *in vivo,* pour mieux élucider les rôles de Gag et de NCp7 dans ce mécanisme. Des expériences étudiant comment APOBEC3G (A3G) empêche la fixation du $tRNA^{Lys3}$ indiquent que dans les virus protéase positifs, la fixation du $tRNA^{Lys3}$ facilitée par Gag peut seulement jouer un rôle mineur. *In vivo* et *in vitro*, A3G empêche seulement la fixation facilitée par la NCp7, et non celle facilitée par Gag. Néanmoins, alors que Gag peut montrer une efficacité de 70-80% de fixation de $tRNA^{Lys3}$ par NCp7 dans des virus protéase-négatif, A3G peut reduire cette efficacité de 40% dans des virus protéase-positifs. Ceci semble être dû au fait que, *in vitro*, la présence de NCp7 rend la fixation antérieurement facilitée par Gag

susceptible à A3G. Ceci suggère que dans des virus du type sauvage, toute fixation du $tRNA^{Lys3}$ à l'ARN viral facilitée par gag est altérée par une refixation par NCp7 sensible à l'A3G.

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appreciation and support I would have never made today. I will always remember the hard times that you had to go through to allow me to accomplish everything I wished for in my life and I will always be proud of you both. Mom and Dad, thank you for everything you have done for me and I love you both dearly.

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Jenan Saadatmand

Preface

In accordance with the guidelines for thesis preparations, the candidate has exercised the option of writing the thesis as a manuscript-based thesis. A general introduction is presented in Chapter 1. Chapters 2-3 are original manuscripts. Chapter 2 currently is in press in the journal "Virology", while Chapter 3 has been submitted. Therefore, chapters 2 and 3 contain their own sections including Abstract, Introduction, Experimental Procedures, Results, Discussion, and Bibliography. A general discussion related to these works and a set of unpublished data has been included in Chapter 4. In Chapter 5, the candidate lists the original contributions to scientific community. In order to link connecting papers, Chapter 2 to 3 each contains a preface.

The manuscripts presented in the thesis are the following:

Chapter II

Saadatmand, J., Fei, G., et al. (2008). Interactions of reverse transcriptase sequences in Pol with Gag and LysRS in the HIV-1 $tRNA^{Lys}$ packaging/annealing complex. (published in Virology (479)).

Chapter III

Saadatmand, J., Niu, M., et al. (2008). Roles of Gag and NCp7 in the annealing of $tRNA^{Lys3}$ to HIV-1 viral RNA. (submitted).

The candidate was responsible for almost all the work presented in this thesis, with the exception of Figure 2.4A presented in Chapter 2, which represents an experiment done by Dr. Fei Guo.

As part of her doctoral training, the candidate also co-authored a number of articles, including:

Zhang, L., **Saadatmand, J.**, et al. (2008). Function analysis of sequences in human APOBEC3G involved in Vif-mediated degradation. Virology. **370** (1): 113-21.

Guo, F., Cen, S., Niu, M., **Saadatmand, J.**, and Kleiman. l. (2006). Inhibition of formula-primed reverse transcription by human APOBEC3G during human immunodeficiency virus type 1 replication. J. Virology. **80**(23): 11710-22.

Halwani, R., Cen, S., Javanbakht, H., **Saadatmand, J.**, Kim, S., Shiba, K., Kleiman, L. (2004). Cellular distribution of Lysyl-tRNA synthetase and its interaction with Gag during human immunodeficiency virus type 1 assembly. J. Virology. **78**(14): 7553-64.

Cen, S., Guo, F., Niu, M., **Saadatmand, J.**, Deflassieux, J., and Kleiman, L. (2004). The interaction between HIV-1 Gag and APOBEC3G. J. Biol Chem. **279**(32): 33177-84.

Cen, S., Niu, M., **Saadatmand, J.**, Guo, F., Huang, Y., Nabel, GJ. And Kleiman, L. (2004). Incorporation of pol into human immunodeficiency virus type 1 Gag virus-like particles occurs independently of the upstream Gag domain in Gag-pol. J Virology. **78**(2): 1042-9.

Javanbakht, H., Halwani, R., Cen, S., **Saadatmand, J.**, Musier-Forsyth, K., Gottlinger, H. and Kleiman, L. (2003). The interaction between HIV-1 Gag and human lysyl-tRNA synthetase during viral assembly. J. Biol Chem. **278**(30):27644-51.

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Abbreviations

a.a.	Amino acid
AaRS	Aminoacyl-tRNA synthetase
AIDS	Acquired immunodeficiency syndrome
ALV	Avian leucosis virus
AMV	Avian myeloblastosis virus
ASV	Avian sarcoma virus
ATP	Adenosine triphosphate
AZT	azidodeoxythymidine
BLV	Bovin leukemia virus
bp	base pair
BSA	bovine serum albumin
CA	Capsid protein derived from HIV-1 Gag precursor
CD4	Cluster determination antigen 4
cDNA	complementary DNA
CXCR4	CXC chemokine receptor 4
CCR5	CXC chemokine receptor 5
DIS	Dimerization initiation signal
DMP	Dimethyl pimelimidate
DNA	Deoxyribonucleic acid
DTT	dithiothreitol reducing agent
EIAV	Equine infectious anemia virus

ENV	Envelope protein
ER	endoplasmic reticulum
ESCRT-1	Endosomal sorting complex required for transport
FIV	Feline immunodeficiency virus
Gag	Group-specific antigen gene
HBV	Hepatitis B virus
HFV	Human foamy virus
HIV-1 (2)	Human immunodeficiency virus type 1 (2)
HTLV	Human T cell leukemia virus
IN	Integrase protein derived from HIV-1 GagPol precursor
KDa	Kilodalton
LAV	Lymphadenopathy associated virus
LysRS	Lysl-tRNA synthetase
LTR	Long terminal repeat
MA	Matrix protein derived from HIV-1 Gag precursor
MHR	Major homology region
MMTV	Mouse mammary tumor virus
MuLV/MLV	Molony Murine leukemia virus
M-PMV	Mason-pfizer monkey virus
MVB	Multi-vesicular body
mRNA	Messenger RNA
NC	Nucleocapsid protein derived from HIV-1 Gag precursor
NLS	Nuclear localization signal

(N)NRTI	(non) nucleoside or nucleotide reverse transcriptase inhibitor
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAS	Primer activation site
PBS	Primer binding site
PCR	Polymerase chain reaction
PPT	Poly purine tract
Pol	Polymerase gene
PR	Protease protein derived from HIV-1 GagPol precursor
$Pr55^{Gag}$	Gag polyprotein Precursor
$Pr160^{GagPol}$	GagPol polyprotein Precursor
RNA	Ribonucleic acid
RNAse	Ribonuclease
RRE	Rev response element
RSV	Rous sarcoma virus
RT	Reverse transcriptase protein derived from HIV-1 GagPol precursor (RNA dependent DNA polymerase, DNA dependent DNA polymerase and Ribonuclease H)
SIV	Simian immunodeficiency virus
SU	Surface HIV-1 glycoprotein
SV40	Simian virus 40
TAR	Trans-activation response element
TM	Transmembrane HIV-1 glycoprotein
tRNA	transfer RNA

U3	3' unique region
U5	5' unique region
5′ UTR	5′ untranslated region
Vif	Virus infectivity factor
VLPs	Viral like particles
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X (HIV-1, SIV)

Chapter I

Literature Review

1.1 Retroviruses

Retroviruses are spherical, enveloped RNA viruses that belong to a large and diverse family called *Retroviridae*. These viruses primarily infect vertebrates. Retroviruses are classified according to structure, composition, and replicative properties. Retroviruses have icosahedral capsids of approximately100 nm diameter. The outer envelope of these viruses is acquired from cell membrane during budding, and inserted into this membrane are viral glycoproteins (Env). The inner core of the virus is composed of capsid (CA) proteins, and within this core is the diploid positive-strand RNA genome coated with nucleocapsid protein, and replicative viral enzymes such as protease, reverse transcriptase, and integrase. These viruses exhibit a unique replicative strategy that includes reverse transcribing their viral genomic RNA into a linear double-stranded proviral DNA by the virally encoded reverse transcriptase enzyme, and subsequently integrating it into the hosts genome (91).

1.1.1.Retroviruses Classification

Based on their genomic organization, retroviruses can be divided into simple and complex viruses (102, 103). In addition to their unique replicative strategy, retroviruses exhibit a variety of other common features. These include a ribonucleic acid (RNA) genome, a common viral structure organized by three major polyprotein precursors, a life cycle involving integration of the viral genome into the hosts' chromosome, and an ability to rapidly mutate their genome in response to environmental cues. All retrovirus genomes contain three major protein-coding domains: *gag*, *pol*, and *env*. Through cleavage by a viral protease, the Gag precursor gives rise to the matrix (MA), the capsid

(CA), and the nucleocapsid (NC) proteins. The Pol precursor gives rise to the reverse transcriptase (RT), integrase enzymes (IN); and the Env precursor gives rise to the surface and transmembrane viral envelope proteins. In addition, the viral protease (PR) may originate from either a GagPro precursor or the Pol precursor, depending on the virus type. All simple retroviruses contain these major proteins, whereas complex retroviruses code for additional regulatory or accessory proteins that are derived from multiply spliced mRNAs. Murine leukemia virus (MuLV) is an example of a simple retrovirus, while human immunodeficiency virus type I (HIV-1) is an example of a complex retrovirus (90).

Previously, retroviruses were subdivided into seven groups based on their evolutionary relatedness (94). Five of these groups belong to retroviruses with oncogenic potential (oncoviruses), and the other two groups are the lentiviruses and the spumaviruses. All oncogenic members except the human T-cell leukemia virus - bovine leukemia virus (HTLV-BLV) genus are simple retroviruses, while lentiviruses (except MLV) and spumaviruses are complex retroviruses.

However, recently, retroviruses were reclassified as those that have simple genomes including: the alpha, beta, gamma and epsilon retroviruses, and those with complex genomes including: the lentiviruses, deltaviruses and spumaviruses (555).

Oncoviruses are tumor-forming viruses that contain an oncogene in addition to the three-retroviral genes, *gag*, *pol*, and *env*. Oncovirinae are further subdivided into five groups (B-type group, mammalian C-type group, Avian leucosis-sarcoma group, D-type group, and HTLV-BLV group). Rous sarcoma virus (RSV) and avian myeloblastosis virus (AMV) are two examples of oncoviruses that belong to the Avian leucosis sarcoma

subfamily.

Lentiviruses are characterized by the slow progression of diseases they cause that are associated with neurological and immunodeficiency disorders. In addition, they are also distinguished by their ability to infect non-dividing cells. Lentiviruses posses a complex gene organization, and HIV and the closely related simian immunodeficiency virus (SIV) are two examples of this family.

Spumaviruses are classically known as "foamy" viruses due to their ability to cause vacuolation in their mammalian host cells. These viruses possess a complex genetic organization and have not been found to be associated with any known diseases (233, 554). Human foamy virus (HFV) is an example of this group.

1.2 Human Immunodeficiency Virus-1 (HIV-1)

HIV-1 belongs to the *Lentivirus* genus, a subfamily of the *Retroviridae*. It is believed to be the agent that causes acquired immunodeficiency syndrome (AIDS). HIV-1 was the first human lentivirus to be identified in 1983 from patients (19) .

1.2.1 HIV-1 Discovery and Evolution

In 1983, the two independent groups that discovered HIV-1 were those of Luc Montagnier from Pasteur Institute in Paris, France and Robert Gallo from the National Institute of Health (NIH) in Bethesda, Maryland (19, 182, 183, 192, 391). Initially, the Luc Montagnier group isolated a new virus from a lymph node of an infected man with persistent lymphadenopathy syndrome and thereby named the virus lymphadenopathy-associated virus or LAV. Robert Gallo's group also reported that they had isolated a human T-cell Leukemia virus HTLV from an AIDS patient, and names it HTLV-III.

Subsequently, LAV and HTLV-III were found to be identical, and their names were changed to HIV (108, 181, 301).

Two types of HIV can be distinguished: HIV-1 (Non-syncytium inducing (NSI) or macrophage-tropic virus (R5) and syncytium inducing (SI) or T-lymphocyte-tropic (X4)) and HIV-2 (syncytium- inducing (SI)). They differ in approximately 55% of the gene sequences, but have similar genes and gene organization (92). HIV-1 is most prevalent in North America, Central Africa, Europe, and Asia while HIV-2 is most prevalent in West Africa (87). HIV-2 is closely related to SIV_{mac} in sequence, and antibodies to both SIV_{mac} and HIV-2 proteins cross react (113, 288). It is believed that both SIV_{mac} and HIV-2 descended from a common ancestor. However, a cross species transmission from primates to human occurred at a later time point (131). In addition, HIV-1 is genetically more diverse, infectious, and transmittable than HIV-2. It is also believed that patients with HIV-2 who have developed AIDS tend to live longer than HIV-1 due to the presence of more neutralizing antibodies in their blood stream (371).

Based on genetic similarities, the numerous HIV-1 strains may be classified into types, groups and subtypes. HIV-1 is divided into three groups: M (main or major), O (outlier) and N (non-M-non-O or new). Group M represents the main group of HIV-1 strains and has been phylogenetically subdivided into different subtypes labeled A, B, C, D, F, G, H, J and K. The genomic sequences of viruses within a subtype are more similar to each other than to sequences of viruses from other subtypes. A great deal of our understanding of the HIV-1 biology and pathogenesis is acquired from the analysis of subtype B, which predominates in Europe and the United States.

1.2.2 HIV-1 Worldwide Prevalence

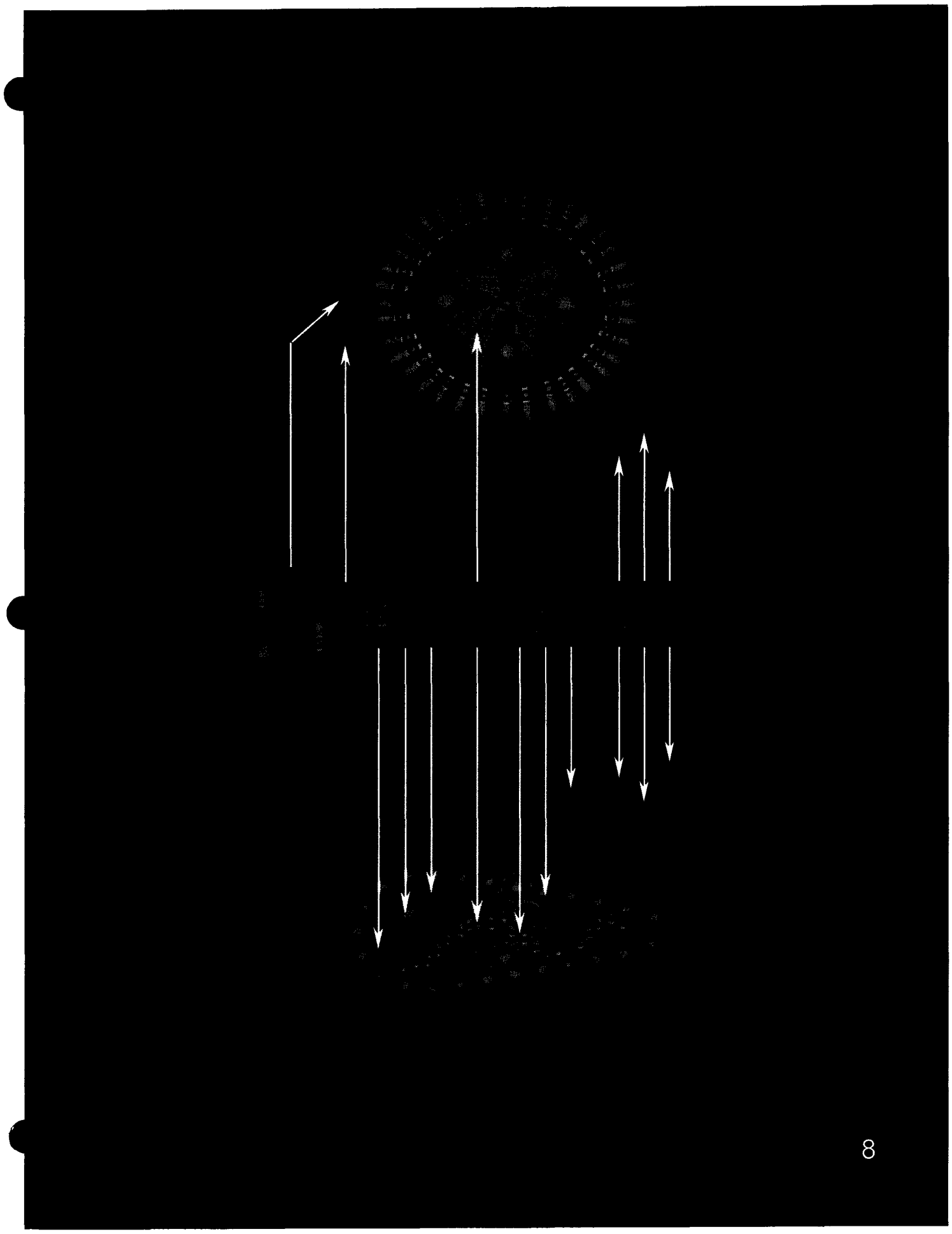
AIDS is one of the foremost causes of death around the world. In 2007, the number of people worldwide living with HIV-1 (HIV-1 prevalence) was 33.2 million, and more than two million people have died from AIDS that year. North America had an estimated adult prevalence of one million persons that are living with HIV (537).

1.2.3 HIV-1 Structure

HIV-1 is an enveloped virus of approximately 110 nm diameter. Figure 1.1 shows a schematic of the HIV-1 viral structure. Its envelope, which is derived from host's cell membrane during budding, contains glycoproteins projections. In the mature particle, the matrix protein (MA) underlines the inner surface of the viral membrane. The viral core is cone-shaped, and is composed of capsid (CA) proteins. Within the core are two molecules of viral genomic RNA, and these are associated with nucleocapsid protein (NC). Also annealed with the viral RNA molecules at the 5′ terminal region is $tRNA^{Lys3}$, the primer for reverse transcriptase in HIV-1. Also found within the core are three virally encoded enzymes involved in the viral life cycle, i.e., protease (PR), reverse transcriptase (RT), and integrase (IN) (91). In addition, many cellular proteins have been found associated with HIV-1, though the function of most of these in the HIV-1 life cycle is unknown (422).

Figure 1.1 Structure of immature and mature HIV-1.

Immature virus (left): HIV-1 assembles at cell membrane, and during budding, becomes surrounded by host membrane. Inserted in the membrane where assembly occurs are the viral envelope (Env) proteins (gp41 and gp120), present in trimeric form. Gag molecules are arranged radially within the virus, with N-terminal MA associated with membrane, and the C-terminal regions of Gag towards center of the virus, in the order CA, NC, and p6. GagPol molecules are also found in the virus with a frequency of 5% the abundance of Gag molecules. Two identical molecules of viral genomic RNA are also found within the virus. **Mature virus (right):** During or after budding, the viral protease is activated, and cleaves the Gag and GagPol precursors, resulting in a change in viral structure. Gag gives rise to MA (underlining membrane), CA (which composes the new cone-shaped core structure), and NC (which coats genomic RNA found within the core). GagPol also gives rise to the viral enzymes, PR, RT, and IN. This diagram does not show cell factors incorporated into the virus during assembly, which can play a role in the viral life cycle.



1.2.4 HIV-1 Genome Organization

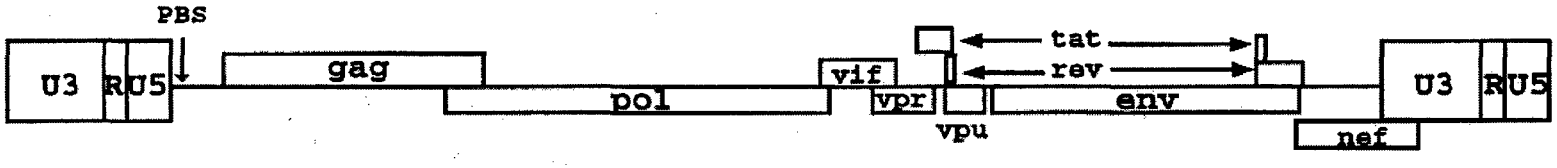
The HIV-1 genome is an RNA of approximately a 9.4 Kb (422), which is found in the virion as a dimer. As described in more detail below, when HIV-1 infects a cell, this RNA is converted to a double-stranded proviral DNA, which then inserts into the host cell genome, where it will be transcribed and spliced into mRNAs that will code for various viral proteins. Figure 1.2 shows the coding and non-coding regions in HIV-1 proviral DNA, viral genomic RNA, and the proteins synthesized by the various mRNAs. The major structural proteins are Gag (a 55 KDa protein, also known as $Pr55^{Gag}$) and Env. The Env precursor protein, $Pr160^{Env}$ is coded for by a spliced 4.3 kb mRNA, and passing through the ER, will be processed by a cellular protease to gp120 and gp41. Gag and GagPol (a 160 KDa protein, also referred to as $Pr160^{GagPol}$), are synthesized from full length viral genomic RNA, with GagPol synthesis resulting from a (-1) ribosomal frame shift occurring after the NC coding region, which results in the synthesis of the Pol part of GagPol in a different reading frame than Gag. The Gag protein contains the amino acid sequences for MA, CA, NC, and a non-structural protein, p6, while the Pol part of GagPol contains the amino acid sequences for PR, RT, and IN (91, 165, 525). Gag and GagPol are processed by the viral protease, which is activated during or after viral budding.

HIV-1 also encodes additional proteins that are referred to as "accessory" proteins, such as Vif (313), Vpr (363) and Nef (280), which are found within the virion and modulate cellular functions for the advantage of the virus. Finally, HIV-1 also encodes two regulatory and expression-enhancing proteins, Tat (437) and Rev (443).

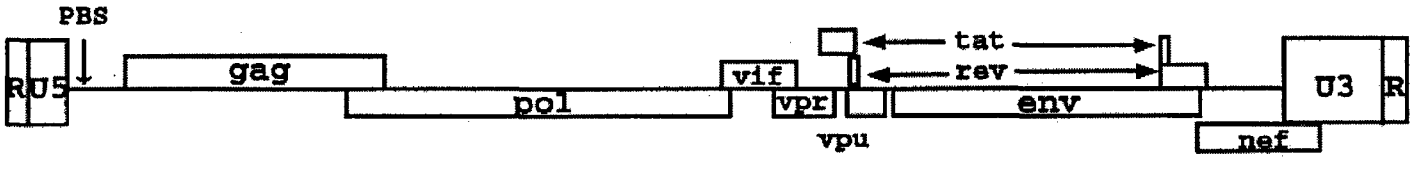
Figure 1.2 Genomic organization of HIV-1 proviral DNA and genomic RNA, and organization of protein sequences within the Gag, GagPol, and Env precursors.

The HIV-1 genome (RNA or proviral DNA) encodes the major retroviral polyproteins Gag, GagPol, and Env, as well as additional regulatory proteins Vif, Vpr, Vpu, Tat, Rev and Nef. The coding region in proviral DNA is flanked by Long Terminal Repeats (LTRs) composed of U3RU5 regions that contain essential regulatory signals for virus replication. Viral RNA transcription starts in the 5′ R (see figure 1.4 for more details on the 5′ LTR region), and terminates in the R region, resulting in viral RNA starting with RU5 and terminating in U3R (Figure 1.5). The *gag* gene codes for viral structural genes including: MA, CA, NC, as well as p6, spacer peptide 1 (Sp1) and spacer peptide 2 (Sp2). The *pol* gene is located downstream of *gag*. Both Gag and Pol are translated from the same mRNA, but translation of Pol RNA sequences occurs in a (-1) reading frame relative to Gag RNA translation, resulting in the GagPol precursor containing sequences for both MA, CA, and NC, as well as the Pol proteins, PR, RT, and IN. The *env* gene codes for the Env precursor protein, whose cleavage by a cellular protease in the ER will give rise to the structural envelope glycoproteins gp120 (SU) and gp41 (TM).

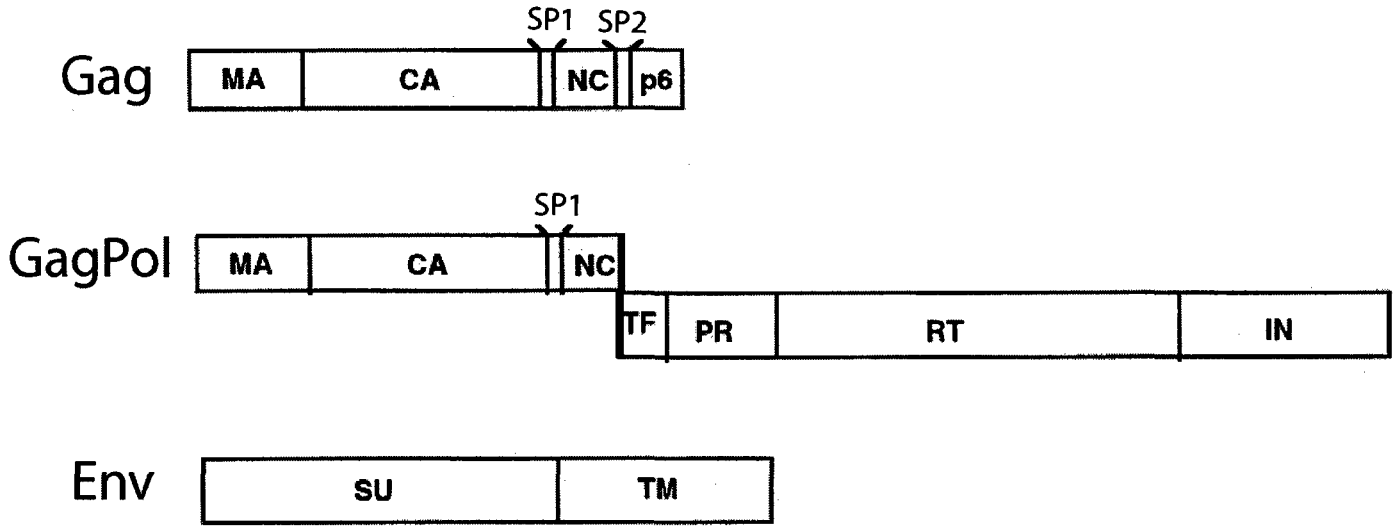
Proviral DNA



Viral Genomic RNA



Viral Structure Proteins



1.2.5 HIV-1 Replication Cycle

HIV-1 infects CD4+ cells, including T-lymphocytes, monocytes, and macrophages. The replication cycle is composed of two phases: infective and replicative. The infective phase is composed of viral entry, conversion of a single stranded RNA genome into a double stranded proviral DNA, translocation of the proviral cDNA into the nucleus, and the integration of this DNA into the host's DNA genome. The replicative phase includes those processes involved in viral RNA transcription, viral protein synthesis, virus assembly, budding, and maturation of the virus.

Figure 1.3 summarizes the various steps in HIV-1 replication cycle. The entry of HIV-1 into the host cell is a multi-step process that is initially mediated by the viral Env protein, and occurs by a pH-independent mechanism (515). HIV-1 attaches via gp120 and infects a target cell expressing the CD4 surface antigen, although other cell-surface molecules have also been confirmed to function as receptors in certain cell types (230, 390). In addition to the CD4 primary receptor, there are various chemokine co-receptors that are used by HIV-1 (316), which will be discussed in more detail in section 1.3.2.4 (Env Proteins).

Once gp120 binds to CD4 receptor, conformational changes are induced in the gp120 subunit, which leads to exposure of a co-receptor-binding site (483). Binding of gp120 to the co-receptor is thought to induce additional conformational changes that lead to activation of gp41, which in turn promotes fusion of the viral membrane with that of the host's (155, 515). Next, the viral core is internalized, and the capsid core is disassembled (93, 515). Envelope proteins and the majority of MA (only 1% of MA proteins are associated with the viral core) are thought to remain at the plasma

membrane (178). While CA is lost, some matrix, NC, IN, Vpr, RT, genomic RNA, and $tRNA^{Lys3}$ are retained as part of high molecular weight complex termed the pre-integration complex. Within this cytoplasmic complex, the viral RNA genome is copied into a double-stranded cDNA by the viral enzyme reverse transcriptase (RT) (164), in a process called reverse transcription, that is initiated from a pre-packaged cellular $tRNA^{Lys3}$ primer (360). The reverse transcription process is discussed in further detail later in this chapter. The resultant viral cDNA is then translocated into the infected cell nucleus and integrated randomly in the host cell's genome by IN (45, 141). Lentiviruses can infect non-dividing cells due to their ability to translocate the preintegration complex into the nucleus, which is facilitated by MA and Vpr proteins (51, 237, 540). The integration process by IN will be discussed later in this chapter. The integrated cDNA or proviral cDNA, codes for viral mRNA and proteins (518). The transcription of the integrated HIV-1 proviral cDNA is regulated by a complex interplay between viral regulatory proteins and cellular transcription factors that interact with the 5′ viral long terminal repeat (LTR) region (Figure 1.4 and 1.5) (11, 279, 473).

The integrated cDNA is transcribed by cellular RNA polymerase II resulting in a 9.4 Kb primary transcript (mRNA) that serves as the full length RNA genome. During HIV-1 replication, three major classes of viral RNA transcripts are produced within the infected cell: unspliced, singly spliced, and multiple or doubly spliced. In the early stage of replication, only the multiple spliced mRNAs enters the cytoplasm and are translated (300). This early class of viral mRNA encodes the viral regulatory proteins Tat, Rev, and Nef (Figure 1.2). Thus, Tat, Rev, and Nef are the first proteins to be synthesized on free ribosomes within the cell's cytoplasm. Following Tat synthesis, it translocates to the

nucleus and binds to the Trans-activation responsive element (TAR) sequence to enhance the transcription of viral RNA (152, 278). Following Rev synthesis, the viral phosphoprotein moves to the nucleus via its nuclear localization signal (NLS)(an Arginine-rich motif)(157). There, it binds to the Rev-response element (RRE) in the viral RNA, a 234-nucleotide RNA structure made up of four stem loops and a long stem within the 3′ region of viral RNA, found within the *env* sequence of full length and singly spliced mRNAs (142, 151, 224, 361, 362). Rev facilitates the translocation of the unspliced and spliced viral mRNA from the nucleus to the cytoplasm by binding to the Rev response element (RRE) RNA with the aid of cellular proteins such as Crm 1, Imp 2, and Ran G-proteins (104, 443). A 4.3 Kb singly spliced mRNA encodes the Env protein and auxiliary proteins Vif, Vpr, and Vpu, while the unspliced 9.4 Kb encodes both $Pr55^{gag}$ and $Pr160^{gagpol}$.

Translation of the Env mRNA occurs in the endoplasmic reticulum (ER), where the $gp160^{env}$ is co-expressed and complexed with the CD4. The $gp160^{env}$ is then proteolyzed by cellular proteases in the Golgi apparatus, yielding two cleavage products: gp120 and gp41 glycoproteins. Release of the $gp160^{env}$ from CD4 (assisted by Vpu) allows the transport of $gp160^{env}$ to the cell surface, where it must be prevented from binding to surface CD4 (a process assisted by Nef). Other viral mRNAs are translated in the cytoplasm. Both Gag and GagPol are translated from the same full-length viral RNA, and this RNA is also packaged into assembling virions where it serves as the viral genomic RNA. Depending upon cell type, Gag and GagPol assemble at the plasma or internal membrane (late endosome), and during, or immediately after, budding from the cell, the viral PR, is activated and cleaves these two precursors into the mature products.

Processing of these precursor proteins by the PR transforms the electron-lucent viral core in the immature particle into the electron-dense viral core structure that is found within the mature virion (421).

During HIV-1 assembly, Gag packages GagPol and two copies of viral genomic RNA. The cellular $tRNA^{Lys}$ isoacceptors ($tRNA^{Lys3}$ and $tRNA^{Lys1,2}$) are also selectively packaged into the virions, and some of the incorporated $tRNA^{Lys3}$ is annealed near the 5′ end of the viral RNA to an 18-nucleotide sequence termed the primer binding site (PBS-Figure 1.4).

After infection of a new cell, the annealed $tRNA^{Lys3}$ is used to prime the RT-catalyzed synthesis of minus strand cDNA, the first step in reverse transcription, which will be reviewed in more detail later in this chapter. For each of these steps in HIV-1 replication cycle, HIV-1 relies on various viral and cellular proteins, which will be discussed in greater detail below.

Figure 1.3 HIV-1 life cycle (Adapted from (302)).

The HIV-1 viral life cycle is depicted in this figure outlining the various essential steps from attachment to budding and maturation of the virus. The packaging of $tRNA^{Lys3}$ and LysRS are also shown since this is a major subject of this thesis. See text for details.

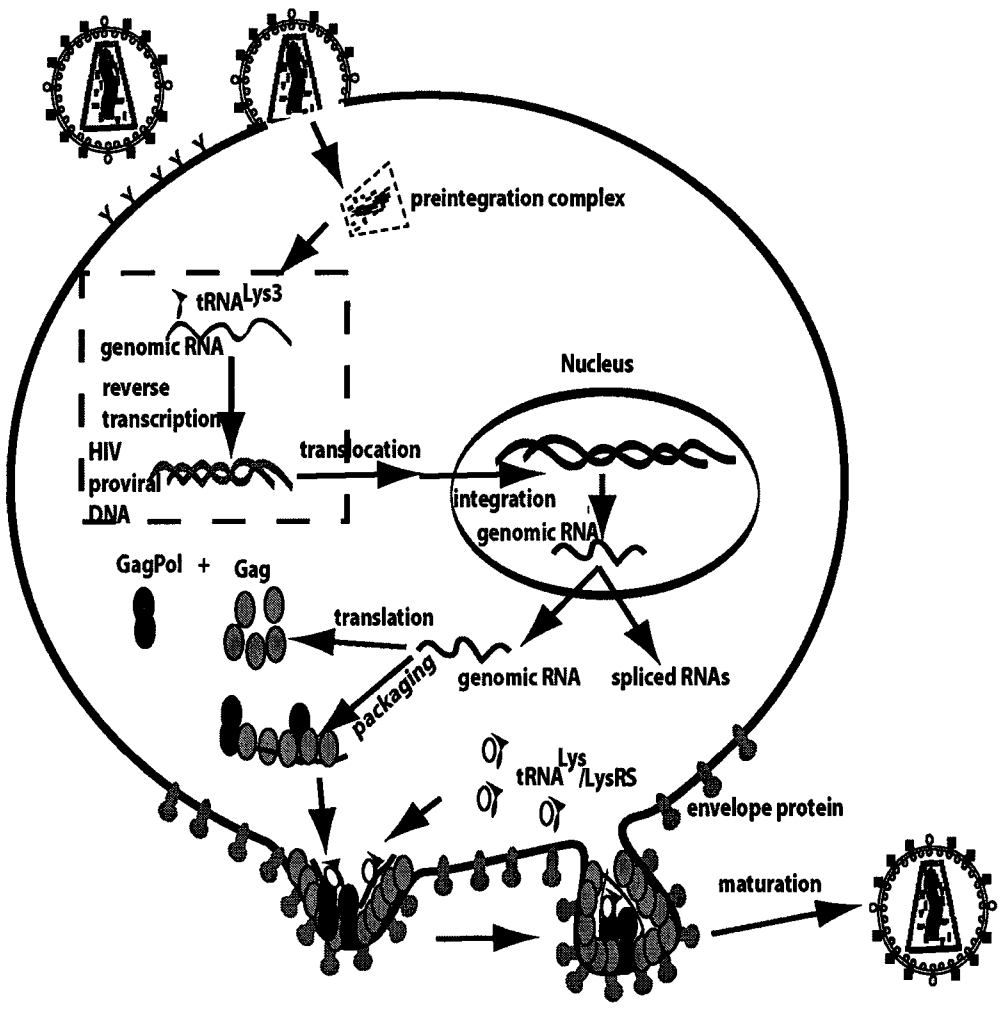
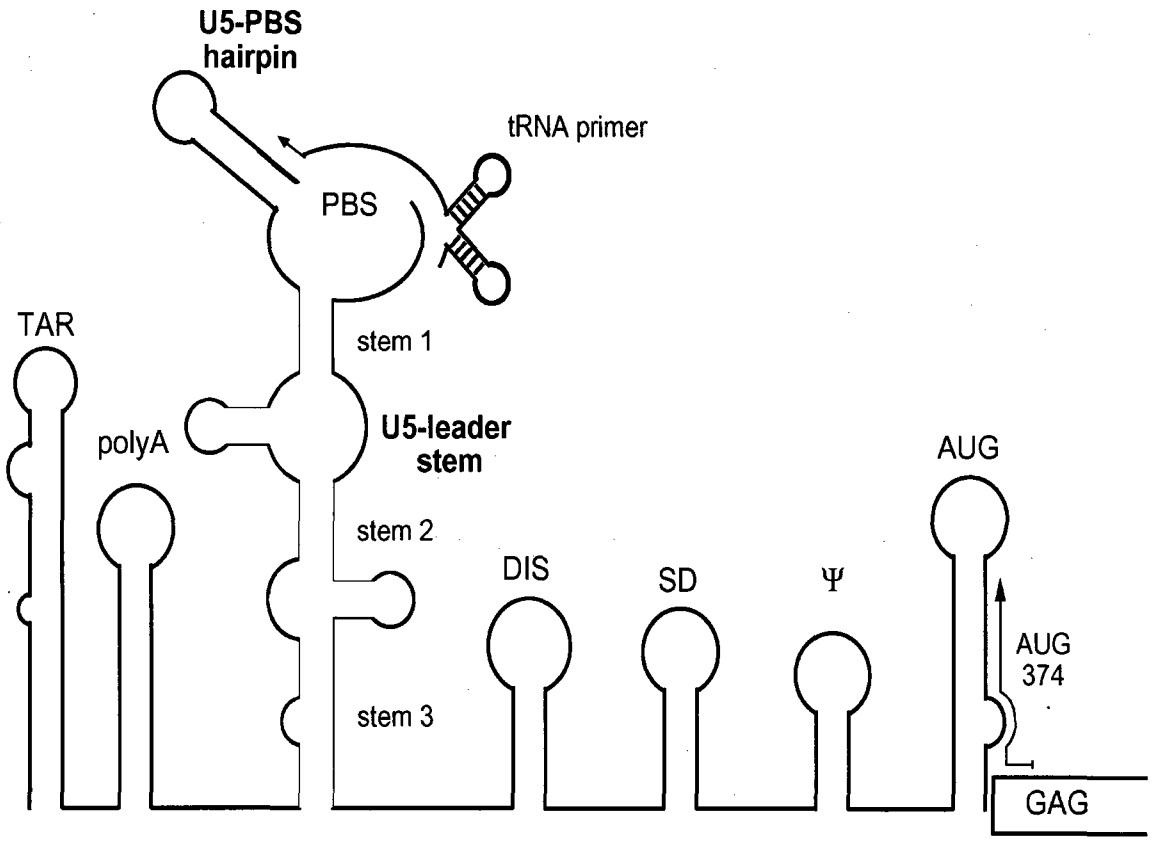

preintegration complex
tRNALys3
genomic RNA
reverse transcription
HIV proviral DNA
translocation
Nucleus
integration
genomic RNA
GagPol + Gag
translation
genomic RNA
spliced RNAs
packaging
tRNALys/LysRS
envelope protein
maturation

Figure 1.4 Sequence of the 5' leader sequence of HIV-1 genomic RNA (Modified from (23)).

The 5′ leader sequence of HIV-1 genomic RNA from R through the AUG initiation codon. The leader RNA contains highly structured stem loop structures that play important roles in transcriptional activation (TAR), mRNA stabililization (Poly (A)), primer $tRNA^{Lys3}$ annealing (PBS), viral RNA dimerization (DIS), and viral RNA encapsidation (PSI, or Ψ). The transcriptional start site is marked with +1 (it marks the border between the upstream (untranscribed) U3 region and the repeat (R) region within the LTR in proviral DNA. The R region (positions +1 to 97) is present at both ends of the RNA genome. The U5 region (positions +98 to 181) is encoded by the LTR and it is unique for the 5′ end of HIV-1 transcript. The leader RNA ends at the AUG start codon of the Gag open reading frame (ORF) (position +336). The primer binding site (PBS) is located at nucleotide positions 182 to 199. The 3' terminal 18 nucleotides of the $tRNA^{Lys3}$ are complementary to the PBS sequence located in the highly structured region at the 5' end of the viral genomic RNA.


U5-PBS
hairpin
tRNA primer
PBS
TAR
stem 1
polyA
U5-leader
stem
AUG
stem 2
DIS
SD
Ψ
AUG
374
stem 3
GAG

1.3 Major Viral Proteins

During HIV-1 life cycle, the major viral structural proteins and enzymes are initially synthesized in the cytoplasm as the Gag ($Pr55^{gag}$) and GagPol ($Pr160^{gagpol}$) precursors, respectively (Figure 1.5). During or after budding, these precursor proteins are processed by viral protease to form the mature viral particle.

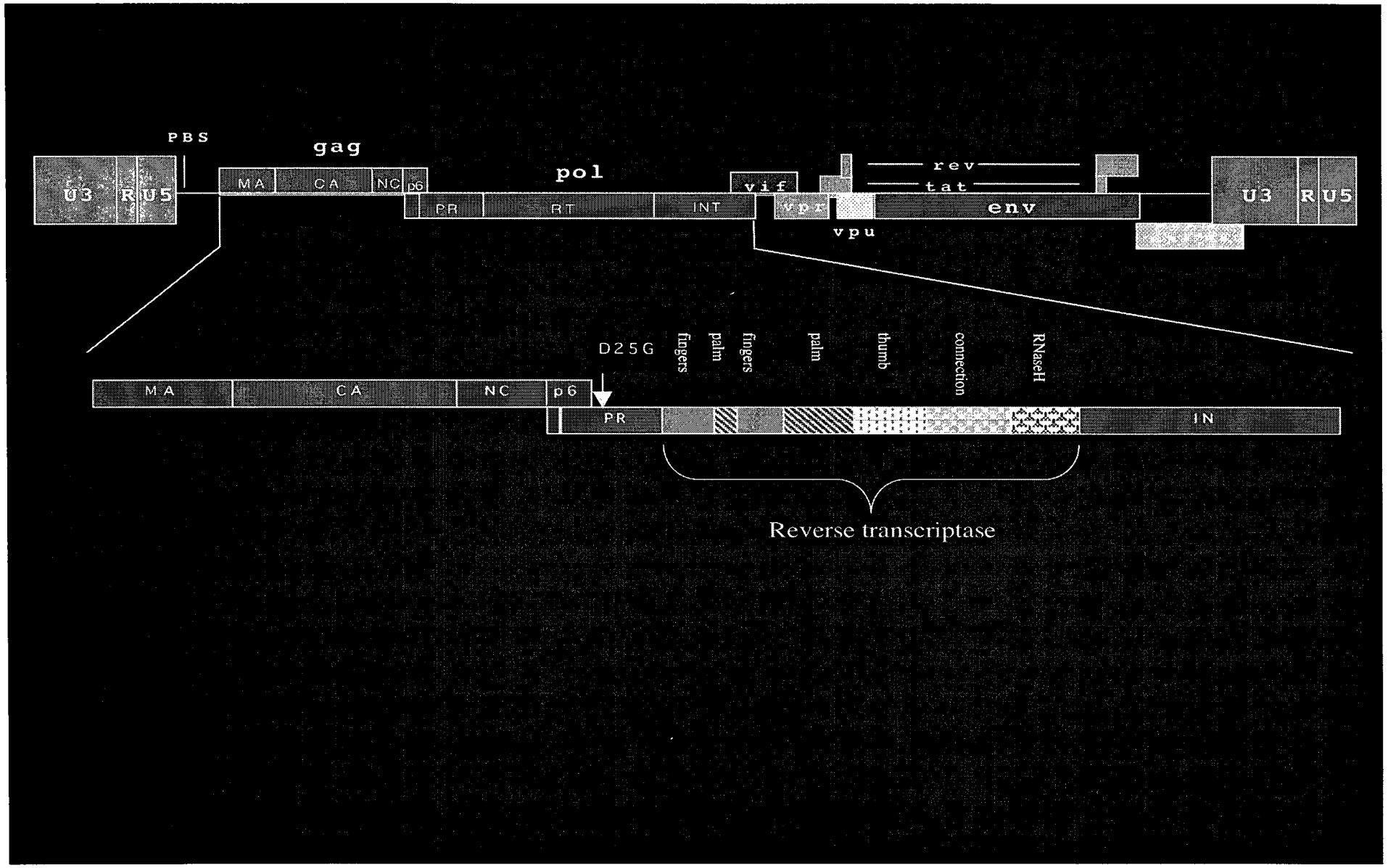
1.3.1 Gag Precursor ($Pr55^{Gag}$) Proteins

The major structural elements of retroviruses are contained in a single 55 KDa polyprotein ($Pr55^{Gag}$). In HIV-1, the unprocessed $Pr55^{Gag}$ is initially synthesized and subsequently proteolyzed by a virally encoded protease to yield the mature proteins: (MA, CA, NC) in addition to the lower molecular mass cleavage products, spacer peptide 1 (SP1), spacer peptide 2 (SP2), and p6 (100, 329). It has been shown that the rate of cleavage of the Gag precursor by the viral protease is somehow regulated by these two spacer peptides, SP1 and SP2 (310, 442, 561). Figures 1.5 and 1.9 illustrate the various protein domains in $Pr55^{Gag}$.

Electron microscopy has shown that Gag is arranged radially in the immature HIV-1 virion, with the N-terminal MA region at the inner face of the membrane and the C-terminal NC-SP2-p6 region nearest to the center (164, 173, 562). For many cell types, Gag assembles at the plasma membrane of the cell, and intracellular expression of Gag alone is sufficient to produce extracellular virion-like particles (VLPs) in the absence of other viral components (194).

Figure 1.5 Schematic representation of HIV-1 proviral DNA depicting the various domains in HIV-1 Gag, GagPol and RT.

The *gag* gene encodes sequences for MA, CA, SP1, NC, SP2, and p6. The *gagpol* gene encode sequences for PR, RT, and IN, in addition to the sequences of MA, CA, SP1, NC). The various subdomains of RT sequences within GagPol are also depicted here, which, based upon the right hand-like appearance of RT, include intervening finger and palm domains, thumb domain, connection domain, and RNase H domain. The (-1) ribosomal frame shift resulting in Pol protein production occurs at the end of NC sequences. D25G is a mutation in PR, rendering it inactive, and is used for the study of PR-negative HIV-1.


PBS
gag
pol
U3
R
U5
MA
CA
NC
p6
PR
RT
INT
vif
vpr
vpu
rev
tat
env
U3
R
U5
D25G
fingers
palm
fingers
palm
thumb
connection
RNaseH
MA
CA
NC
p6
PR
IN
Reverse transcriptase

1.3.1.1 Matrix (MA)

HIV-1 matrix (MA) protein is the N-terminal cleavage product of $Pr55^{Gag}$. MA has a molecular weight of 17 KDa and is found as a trimer. MA underlines the inner surface of the viral membrane. Both NMR and X-ray crystallography have elucidated the structure of MA (97, 243, 376). The MA protein folds into a compact core domain, which consists largely of α–helices and a three stranded β–sheets (536) .

MA protein has been suggested to participate in several important roles of the virus replication cycle: 1) The efficient anchoring and incorporation of the Env (TM) protein into budding virions (133, 168, 170, 364, 580); 2) The specific targeting and directing of Gag to the cell membrane (47, 164, 171, 205); 3) The nuclear localization of the pre-integration complex (51, 179); 4) Binding to the viral genomic RNA (50).

HIV-1 MA contains a nuclear localization signal (NLS) and a myristoylation signal (which serves as a membrane localizing signal) within the N-terminal first 50 amino acids that are crucial at different stages of the virus life cycle (51, 178). It has been shown that MA is cotranslationally myristoylated and serves to anchor Gag to the cell membrane (243, 376, 478). In most retroviruses, the myristic acid moiety is attached to the N-terminal glycine residue of the Gag protein (495). The covalent attachment of myristate to the N terminus of the MA domain of Gag precursor is critical for the binding of Gag to membrane and is hence required for virus assembly (47, 194, 205, 311, 428, 565).

In addition to myristate, basic amino acids in the highly basic domain at the N-terminus and other residues at C terminal domain of MA may also facilitate Gag binding to membrane by promoting an electrostatic interactions with acidic phospholipids in the

inner leaflet of the membrane (376, 592). MA has also been implicated in the targeting of virus assembly at different cellular locations because mutations in MA can cause either indiscriminate virus assembly both on the plasma membrane and at intracellular sites (324, 394, 454, 547), or redirection of assembly to intracellular locations such as the cytoplasm, Golgi or ER (149, 180, 418, 512). Amino acid substitutions near the N terminus of MA have also been reported to reduce infectivity in single round assays and interfere with the synthesis of viral DNA post-infection (63). In addition, it has been demonstrated that the C-terminal of MA has a role in virus replication and DNA synthesis in infected cells (578).

1.3.1.2 Capsid (CA)

HIV-1 CA protein is the major structural subunit of the mature viral core, with about 2000 molecules per virion (186). It has a molecular mass of 24 KDa. *In vitro* studies have shown that purified CA has intrinsic properties of self-assembly *in vitro* due to its ability to form dimers and oligomeric complexes under appropriate pH conditions (58, 139, 196, 387). NMR and X-ray crystallography have been used to deduce the structure of HIV-1 CA, and have shown that it forms dimers in solution (185, 389). Electron microscopy studies of immature virions have shown that Gag is arranged in interacting hexagons, with a Gag at each vertex of the hexagon. Hexagonal order appears to reside mainly at the level of C terminal domain of CA and Sp1 (41, 42, 378, 401). In the mature virion, the CA again forms a hexagonal lattice within a conical capsid closed by 12 pentamers (43, 187, 336).

The CA is structurally subdivided into two domains: an N-terminal domain which functions in virion core maturation and incorporation of cellular protein

cyclophilin A (CypA)(163, 184, 351, 424, 532), and a C-terminal domain that contributes to Gag/Gag interaction and contains the binding site for lysyl-tRNA synthetase (LysRS)(274, 307, 308). Thus, various studies have shown that the CA N-terminal domain is not required for assembly; however, the CA C-terminal domain is essential for immature particle formation (2, 7, 9, 38, 310).

The CA C-terminal domain is crucial for CA dimerization. It has been shown that small deletions at the CA C-terminal end resulted in a reduction in virus production. Interestingly, viral assembly can be achieved using a minimal Gag construct that is composed of the Gag myristylation site, the C-teminal region of CA, a dimerization domain from a yeast factor replacing NC, and p6 (2). Although mutations within the N-terminus of the CA does not prevent particle assembly, they do result in the production of viruses with reduced infectivity (2).

It has been shown that Gag binds LysRS. The domains critical for the interaction between Gag/LysRS have been mapped to include the dimerization domain in CA within $Pr55^{Gag}$ (274) (Figure 1.9 A). In fact, a recent *in vitro* work indicated that monomeric CA interacted specifically with LysRS, with k_D = 400 nM (307). The same group have fine- mapped the critical interacting region to be helix 4 within the C-terminal domain of CA (308)(Figure 1.9B).

It has been demonstrated that CA contains the only region of Gag that displays significant homology among many different genera of retroviruses. This region, the major homology region (MHR), plays a role in virion morphogenesis (365, 565). The MHR has been demonstrated to be essential for viral particle assembly and appears to be required for $Pr160^{GagPol}$ incorporation into virion (513).

1.3.1.3 Nucleocapsid (NC)

NC, also known in HV-1 as NCp7, is a nucleic acid binding protein with a 7 KDa molecular weight that is found tightly associated with the viral RNA within the viral core. NC has been suggested to be involved in a number of retroviral process, including: 1) genomic RNA packaging (107, 470); 2) RNA dimer formation (283, 320), 3) stability of the preintegration complex (164), 4) strand transfer of minus strong stop cDNA in reverse transcription (114, 216, 481), 5) primer tRNA annealing (119, 120, 254, 455), and 6) Gag-Gag interactions (242).

The NC polypeptide contains two motifs, termed zinc fingers, which contain cysteine and histidine (Cys-His) residues. This Cys-His motif has the structure Cys-X2-Cys-X4-Cys-His-X4-Cys (CCHC), and is highly conserved among all retroviruses, except in spumaviruses, but the majority of the Xs are not conserved among retroviruses (377). While basic amino acid regions flanking the first zinc finger are believed to facilitate the non-specific binding of NC to RNA, the zinc fingers are believed to play a role in the specific binding of NC to HIV-1 genomic RNA (28), through specific interactions with a 120 nucleotide region in the 5′ regions of viral RNA known as the RNA leader sequence (88) (Figure 1.4), that contains various stem loop structures, of which stem loop 3 (SL3) seems prominent in contributing to the RNA packaging signal, also known as Ψ (235) . However, other stem loops in this region could also be important (89, 380).

RNA dimerization is also believed to be facilitated by NC through its promoting annealing of certain sequences in the leader sequence, that include the site of initiation of

dimerization, SL1, which contains the so-called kissing loop (see Figure 1.4). Specific basic amino acid residues within NC that flank the first zinc finger are important for HIV-1 RNA dimerization (115), and it has been recently shown that the amino-terminus, the proximal zinc finger, the linker, and the distal zinc finger of NC each contributed roughly equally to efficient RNA dimerization (283).

$tRNA^{Lys3}$ annealing to viral RNA also requires the presence of basic amino acid residues flanking the first zinc finger in NC (318). It is believed that NC's role in this is to both promote nucleation of $tRNA^{Lys3}$ and viral RNA, and to unwind double stranded regions in viral RNA that are involved in this annealing (266). The function of NC in promoting annealing will be reviewed in more detail later in this chapter.

NC also facilitates DNA strand exchange, where newly-synthesized minus strand strong stop cDNA is transferred from the 5′ to the 3′ends of the RNA genome, which occurs during reverse transcription (218). The role of NC in this step will be discussed in further detail later in this chapter.

NC sequences in Gag also interact with Human Apolipoprotein B mRNA-Editing Enzyme-Catalytic Polypeptide-Like-3G (APOBEC3G) (8, 65, 491, 584). The role of this interaction in facilitating APOBEC3G incorporation into Gag VLPs, and how it affects $tRNA^{Lys3}$ annealing, will be discussed further in this chapter and chapter 3 of this thesis.

NC also plays an essential role in retroviral immature particle formation through promoting Gag self-association and assembly (2, 9, 83, 84, 484, 485, 588). Studies performed both *in vivo* and *in vitro* have indicated that in addition to its direct role in facilitating Gag/Gag interaction, NC-facilitated binding of Gag to RNA may result in RNA acting as a scaffold to promote Gag/Gag interaction. However, replacement of NC

with a yeast leucine zipper motif that is a protein-protein interaction domain can replace the Gag assembly function of NC with this leucine zipper domain, and result in a mutant Gag capable of assembling into extracellular VLPs (2).

1.3.1.4 p6

p6, a 51 amino acid (a.a.) peptide, is the C-terminal cleavage product of Gag. Within p6, a critical region composed of four amino acids (Pro7, Thr8, Ala9, Pro10) is called the L (late) domain or PTAP, and this region has been observed to play a role in assembly and efficient release of virus (204, 253). It was shown that the p6 late domain interacts with cellular Tsg101, so as to facilitate virus budding (188, 373, 539). In uninfected cells, Tsg101 was shown to function in the biogenesis of the multivesicular body (MVB) that is involved in the endocytic trafficking pathway, and which is required for the sorting of mono-ubiquitinated transmembrane proteins into internal vesicles (15, 136, 335, 539). HIV-1 maturation and budding is dependent on Tsg101 expression (188, 373). After the binding of the cellular Tsg101 to the late domain in p6, Tsg101, which is a component of the ESCRT-1 complex (endosomal sorting complex required for transport), recruits multiple components of the MVB machinery into the site of retrovirus assembly in order to facilitate viral particle budding into the extracellular environment (10, 138, 198, 395, 521, 523, 543). In addition, p6 contains a binding site for Vpr, and it has been shown that Vpr is incorporated into the virus through binding to p6 (306, 350).

Recently, p6 sequences have been shown to be required for an interaction between Pol and Gag (72, 342), which will be discussed in more detail in chapter 2.

1.3.2 GagPol Precursor ($Pr160^{gag\text{-}pol}$) Proteins

$Pr160^{gagpol}$ is a 160 KDa fusion protein that encodes the viral structural proteins MA, CA, and NC, as well as the three viral enzymes that are encoded by the *pol* gene, (PR, RT, and IN). Figure 1.5 depicts the various protein domains in GagPol.

During translation of the full length viral mRNA, $Pr160^{gag\text{-}pol}$ arises from a minus one (-1) ribosomal frame shift event near the C-terminus of the *nc* gene. The ribosome slips backward one nucleotide, thereby eliminating the Gag stop codon, and continues to translate *pol* sequences (269, 567) (Figure 1.2 and 1.5). Frame shifting occurs 5% of the time, i.e., about one GagPol molecule is synthesized for every 20 Gag molecules. Frame-shifting is mediated by a heptanucleotide slippery sequence found at the site of the frame shift (567) and a downstream hairpin (135). GagPol is incorporated into Gag viral particles during HIV-1 assembly via a Gag/GagPol interaction.

1.3.2.1 Protease (PR)

HIV-1 PR is an 11 KDa aspartyl protease that is responsible for the proteolytic cleavage of the newly synthesized viral $Pr55^{gag}$ and $Pr160^{gagpol}$ that results in the production of mature viral proteins and virus (305). PR exists as a homodimer with two identical monomers of 99 amino acid residues with identical conformation. Each monomer contributes one of the active catalytic site that is located at the junction of Asp-25 and Asp-25′. It is structurally related to cellular aspartic proteases such as pepsin, but cellular proteases are distinct in that they are monomers, with two domains that resemble the structure of monomers of HIV-1 PR. In addition, the PR active site

structurally resembles other aspartic acid proteases in that it contains a highly conserved catalytic triad sequence Asp-Thr-Gly (348).

Previous studies have shown that dimerization of the PR is required for its activation. Once dimerization occurs, PR is autocatalytically cleaved out of the GagPol (565). Hence, PR activity depends on GagPol concentration. It has been suggested that the N-terminal sequences upstream of the PR effect autoprocessing. In addition, the SP1 spacer peptide 1 located between CA and NC may also influence cleavage rates and viral infectivity (356, 432). Furthermore, it has been shown that overexpression of PR leads to aberrant processing rates and decreased infectivity, due to the fact that assembly and maturation must be coordinated (356). Once the fully active PR dimer is formed, it cleaves the Gag and GagPol into mature viral proteins. Site directed mutagenesis studies have shown that a mutation in the protease gene rendered HIV-1 virus non-infectious and immature, i.e., the viruses produced contained unprocessed Gag and GagPol (305, 348, 366). While it was initially believed that precursor processing by the PR occurred only after viral budding, it has now been shown that efficient viral budding requires the proteolytic cleavage of viral precursors to be initiated at the membrane of infected cells simultaneously with assembly and budding (164, 290).

PR is a prime target in drug design due to its importance in the HIV-1 life cycle. Many PR inhibitors have been approved for clinical use in the treatment of AIDS, and in combination with other inhibitors, have proven to be successful in treatment of HIV-1 infection. However, mutants resistant to multiple PR inhibitors have been found (96, 464). These resistant mutations have been found both within the binding pocket of the inhibitors and at distant sites. Interestingly, some mutations show increased catalytic

activity (494).

1.3.2.2 Reverse Transcriptase (RT) and the Reverse Transcription Process

1.3.2.2.1 RT Structure and Properties

Mature HIV-1 reverse transcriptase (RT) enzyme is a multifunctional, heterodimer (66 and 51 KDa) consisting of two subunits, p66 and p51 (344). Formation of p66 homodimers is followed by viral protease-facilitated cleavage of one subunit (removing the RNaseH domain) to produce the p66/p51 heterodimer (250, 251). RT belongs to a diverse of group of enzymes, the DNA polymerase superfamily. However, RT is special among these polymerases in that it can utilize both RNA and DNA as template. In addition, another distinct feature of RT is its lack of proofreading ability.

RT possesses three distinct functions: an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase, and a ribonuclease H (RNase H) function. These functions enable it to synthesize the proviral double-stranded DNA from a single stranded viral genomic RNA template during the HIV-1 life cycle (91). Both the DNA polymerase and the RNase H activities reside within the large p66 subunit of RT (249, 321, 323).

The overall structure of the enzyme has been determined by X-ray crystallography. Based on its similarity to the profile of a right hand, RT has been divided into distinct domains termed the fingers, palm, thumb, and connection domains, and a separate RNase H domain (Figure 1.5). The active site for the polymerization site is located in a cleft of the palm, between the fingers and the thumb (270, 271, 468, 530). The RT catalytic site lies in the cleft of p66 subunit and it contains three residues (Asp-

185, Asp-186 and Asp-110). The connection domain holds the RT and C-terminal RNase H domains together (271). The p51 subunit is similar to the p66 in sequence except that it is inactive and it lacks the RNase H domain of p66. The interaction between both subunits of RT is required for optimal enzyme activity. It has been shown that monomeric or homodimeric enzymes formed with either p51 or p66 have reduced activity compared to p51/p66 heterodimer (514).

As we shall see later, RT is involved in the selective packaging of $tRNA^{Lys3}$ into virions, and its annealing to the viral RNA (116, 296, 341, 358, 359, 425, 440, 488). RT sequences within GagPol are one of the essential components required within the $tRNA^{Lys}$ packaging/annealing complex, which will be discussed in more detail later in this chapter. We will also provide evidence in chapter 2 for an interaction occurring between RT and LysRS.

1.3.2.2.2 RT Incorporation into HIV-1

While normal incorporation of RT into HIV-1 occurs as a result of GagPol incorporation, RT can be incorporated into viral particles. A study by Liao *et al.* has shown that HIV-1 RT sequences alone are incorporated into Gag VLPs, with apparent interactions occurring between matrix and p6 sequences in Gag and the thumb domain in RT (342). In chapter 2, which focuses on the interaction between LysRS and RT, we provide further evidence that HIV-1 RT alone can be incorporated into Gag VLPs via an interaction with Gag, supporting the findings of Liao *et al.*

1.3.2.2.3 The Reverse Transcription Process

Reverse transcription is a complex and multi-step process that includes strand

displacements. During retroviral reverse transcription; HIV-1 RT uses a prepackaged $tRNA^{Lys3}$ as a primer and the viral genomic RNA as a template to initiate the reverse transcriptase-catalyzed synthesis of minus strand strong stop DNA (93). The packaging of $tRNA^{Lys3}$ into HIV-1 during viral assembly requires RT sequences within GagPol (296). The 3' terminal 18 nucleotides of this $tRNA^{Lys3}$ are complementary to the primer binding site (PBS) sequence located in the highly structured leader region at the 5′ end of the viral genomic RNA (452). Figure 1.4 shows the 5' leader sequence of the HIV-1 genome RNA.

Figure 1.6 illustrates the various complex steps involved in reverse transcription process, which occur within the preintegration complex immediately after infection (328, 530, 531). The first step in reverse transcription is the production of a minus strand strong stop DNA (-) ssDNA, the first discrete intermediate in retroviral DNA synthesis (234, 554). During its synthesis, the RNA template giving rise to (-) ss DNA is degraded by RNaseH (34). This facilitates the first template switch, when (-) ss DNA is transferred to the 3′ end of the same RNA template via annealing of the sense R sequence and the anti-sense R sequence in the (-) ss DNA. Further reverse transcription produces (-) ssDNA that advances past the end of the PBS sequence on the viral genomic RNA. During this process, all but two small stretches of RNA sequences known as the poly purine tract in the U3 region (3′-PPT) and the center of the template (central PPT, cPPT) in the viral genomic RNA are degraded by RNase H. These RNase H resistant RNA regions are used to prime the synthesis of the plus strand strong stop DNA (+) ssDNA, resulting in the synthesis of the U3, R, U5, and PBS regions, with reverse transcription stopping after transcription of the 3′ terminal 18 nucleotides of the

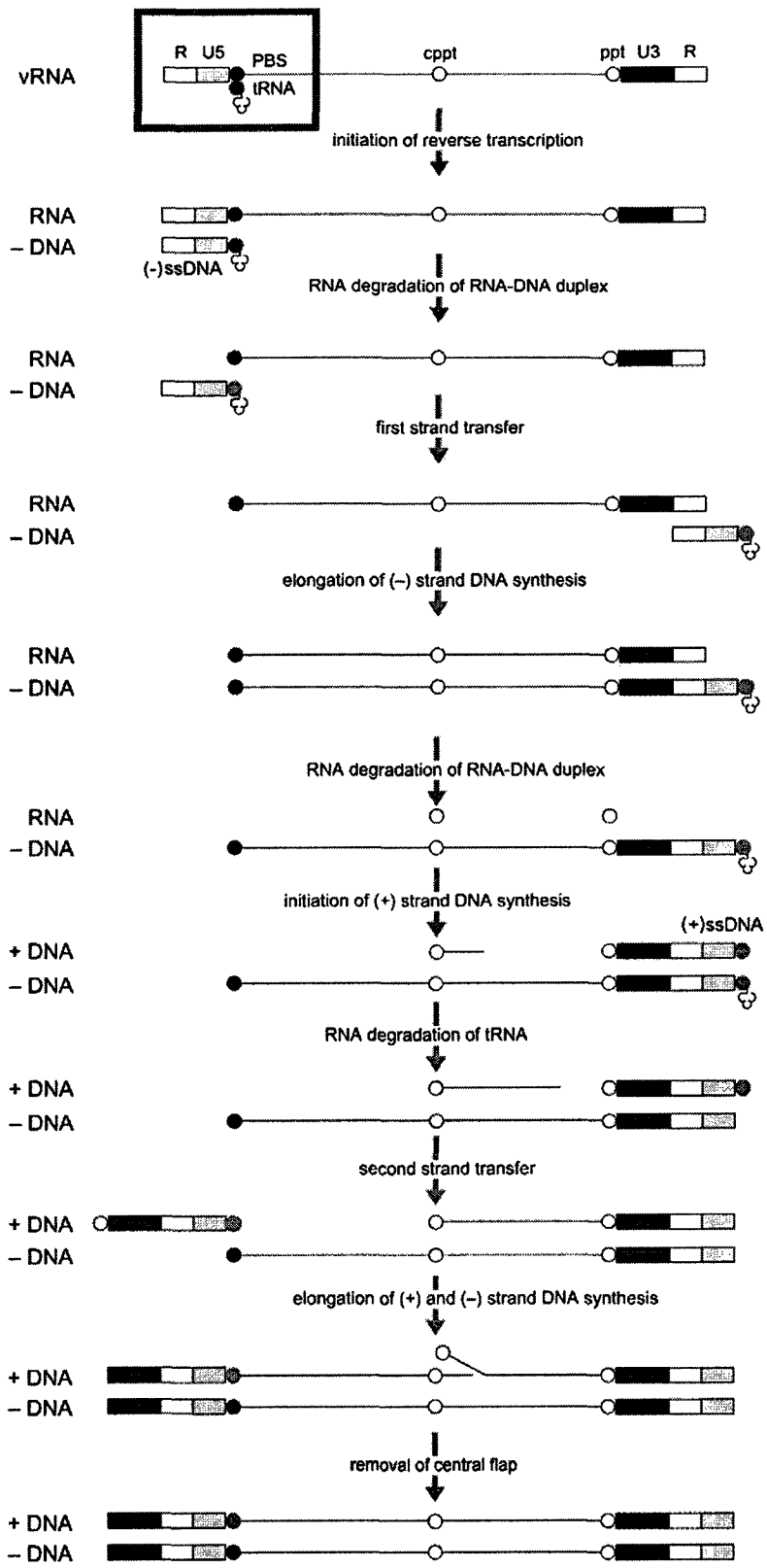
$tRNA^{Lys3}$ (1).

A second template switch of this plus strand DNA then occurs, and the newly synthesized PBS region in the 3′ end of the (+) ssDNA anneals to the complementary sequences located in the 3′ end of the elongated (-) ssDNA. Reverse transciption proceeds over the (-) ssDNA until it encounters the cPPT-extended (+) ssDNA. Elongation occurs through the cPPT via a mechanism called strand displacement until RT reaches a nearby site (80–100 nts downstream of the cPPT): the central termination sequence. This motif is very efficient in terminating HIV-1 RT-catalyzed DNA elongation (1). (+) ssDNA and (-) ssDNA continue to be synthesized yielding a full-length, double-stranded, proviral DNA that is flanked by two identical long terminal repeats (LTRs) composed of U3, R, and U5 regions and contains a discontinuous plus strand DNA with a approximately 99 bp DNA flap at its center (531).

This central DNA flap is a three stranded structure with two overlapping positive-strand segments and its synthesis is known to be required for HIV-1 replication in non-dividing cells (76, 223, 259, 517). The central flap is removed by a cellular endonuclease and DNA ligase completes the continuous double strand DNA (476).

Figure 1.6 The HIV-1 reverse transcription process (Adapted from (1)).

See text for details.


vRNA
R U5
PBS
tRNA
cppt
ppt U3 R
initiation of reverse transcription
RNA
– DNA
(-)ssDNA
RNA degradation of RNA-DNA duplex
first strand transfer
elongation of (–) strand DNA synthesis
RNA degradation of RNA-DNA duplex
initiation of (+) strand DNA synthesis
(+)ssDNA
+ DNA
RNA degradation of tRNA
second strand transfer
elongation of (+) and (–) strand DNA synthesis
removal of central flap

1.3.2.2.4 RT Inhibitors

Conversion of the HIV RNA genome into DNA by viral RT is a key step in the early stages of the HIV life cycle, making the enzyme an ideal target for antiretroviral therapy. Two classes of RT inhibitors are available: nucleoside or nucleotide reverse transcriptase inhibitors (NRTI's/NtRTI's) and non-nucleoside reverse transcriptase inhibitors (NNRTI's). The NRTI's were the first antiretrovirals to be utilized for the treatment of HIV. Based on their resemblance to the natural nucleotide building blocks of DNA and RNA, NRTI's are incorporated into the growing DNA strand thus terminating further strand elongation. On the other hand, NNRTI's are a chemically diverse class of drugs that bind to the same pocket near the active site of RT and by so doing, they inhibit the RT enzyme.

Collectively, these drugs are known as RT inhibitors and include the nucleoside and nucleotide analogues zidovudine (AZT, trade name Retrovir)(158), lamivudine (3TC, Epivir)(319) and tenofovir (Viread)(469), as well as non-nucleoside inhibitors, such as nevirapine (Viramune)(208) and Efavirenez (Sustiva)(4). Unfortunately, the RT enzyme is characterized by low fidelity for nucleotide incorporation due to the absence of 3′ exonuclease activity that renders the enzyme error prone (433, 445, 530). Therefore, rapid mutations give rise to RT's that are resistant to RT inhibitors due to the high error rate of RT and its lack of proof reading ability.

1.3.2.3 Integrase (IN)

IN is an enzyme with a 34 KDa molecular mass that is responsible for the integration process of the double stranded proviral DNA into the host genome (52, 463).

The IN and the newly synthesized proviral cDNA are parts of the preintegration complex (52). IN has been shown to recognize the LTR at the 5′and 3′ ends of the newly synthesized proviral cDNA, and cleave 2-3 bases from the 3' ends. This cleavage reaction creates recessed hydroxyl groups at the 3′ terminus waiting to be ligated to the host cell genome in the nucleus. Furthermore, the pre-integration complex is translocated into the nucleus by the aid of two other viral proteins called the MA and Vpr (51, 237, 540). In the nucleus, the IN then joins the 3′ ends of the proviral cDNA to the host cellular DNA (45, 141). DNA synthesis is then followed to fill gaps in the unligated 3′ ends of the cellular DNA, yielding the complete integrated proviral cDNA (45).

IN is found as tetramers (463) and structurally is divided into three functional domains: the N-terminal domain promotes IN multimerization (55, 325, 589), the catalytic domain promotes enzymatic activity (463) and the C-terminal domain promotes nonspecific DNA binding (140, 143, 148). The structures of each domain have been individually determined by X-ray crystallography or NMR spectroscopy. Currently no crystal structure data exists with IN bound to its DNA substrates due to the insoluble nature of IN (147).

Besides the function of IN in the integration process of the viral DNA into the host genome (315, 568), it appears to play a role in other aspects of the HIV-1 life cycle such as: reverse transcription (572), nuclear import of the preintegration complex (39, 177), HIV-1 particle production (49), and polyprotein processing, assembly, and maturation (49, 448, 500).

A specific interaction has also been reported to occur between HIV-1 IN and RT (572), where an *in vitro* interaction between the finger/palm domain and the carboxy-

terminal half of the connection subdomain of RT and the C-terminal domain of IN was observed (236). It is not known if such an interaction also occurs in full-length GagPol. Another group has shown that IN plays an important role during the reverse transcription step of the viral life cycle, possibly through its interactions with RT (594). The same group has also reported that IN acts in the early steps of reverse transcription by increasing RT enzyme processivity and suppressing the formation of the pause products (127).

IN is an attractive target for new anti-HIV drugs. Currently, there is only one approved IN inhibitor Raltegravir (Isentress^{TM}, trade name) for treating HIV infections (490).

1.3.2.4 Envelope (Env) Proteins

HIV-1 envelope (gp160^{env}) is translated from a singly spliced RNA that is proteolytically processed in the secretary pathway by a cellular protease into two mature subunits: a receptor binding subunit (SU or gp120) and a transmembrane subunit (TM or gp41) (129, 260). These proteins remain associated through non-covalent interactions (Figure 1.2). Env glycoproteins play important roles in viral entry and receptor binding to host cells (25, 75, 169, 392). The gp120 glycoprotein is found as trimer or tetramer (i.e. three SU gp120 and three TM gp41 molecules) (137, 426, 492, 509, 553) and is required for the initial binding of the virus to the primary cellular CD4 receptor on target T cells lymphocytes (108, 314). The gp120/CD4 interaction triggers a series of conformational changes in gp120 that eventually exposes a binding site for a cell surface chemokine receptor such as CCR5 or CXCR4 that acts as a co-receptor (482). CXCR4 was the first co-receptor to be identified, which permits entry of T-tropic, synctium

inducing (SI) viruses, i.e., viruses that infect T-cells but not macrophages (153). Later, CCR5 was discovered. It is a major co-receptor for M-tropic, non-syncytium inducing (NSI) viruses, i.e., viruses that infect macrophages but not T cells) (124).

In addition, studies have shown that other molecules that act as co-receptor for HIV-1 also exist (86).

Upon binding, conformational changes occurr in gp120 that initiate the membrane fusion activity of the transmembrane gp41 glycoprotein (314, 466). The conformational change in gp120 leads to the exposure of a hydrophobic N-terminal peptide sequence (20 a.a.) of gp41, which initiates the fusion of the viral and host cell membranes (25, 155, 573).

1.4 HIV-1 Assembly

1.4.1 Patterns of Retroviral Assembly

Retroviruses are classified into four types based on their morphological characteristics and the cellular location of capsid formation (525, 529). These four types include type A, type B, type C, and type D. Figure 1.7 illustrates the different patterns of retroviral assembly.

Type A virus form spherical intracellular particles containing immature cores, with the capsid assembling within the cytoplasm and remaining inside the cells. These immature particles assemble at and bud into the membranes of the ER, where they remain as enveloped spherical particles of unprocessed Gag. Type A morphogenesis is abundant in many murine tumors and is also found in normal tissues of mice and other rodents. These type A particles act as precursors for some type B particles and resemble

the assembly pattern for the immature intracellular form of mouse mammary tumor virus (MMTV).

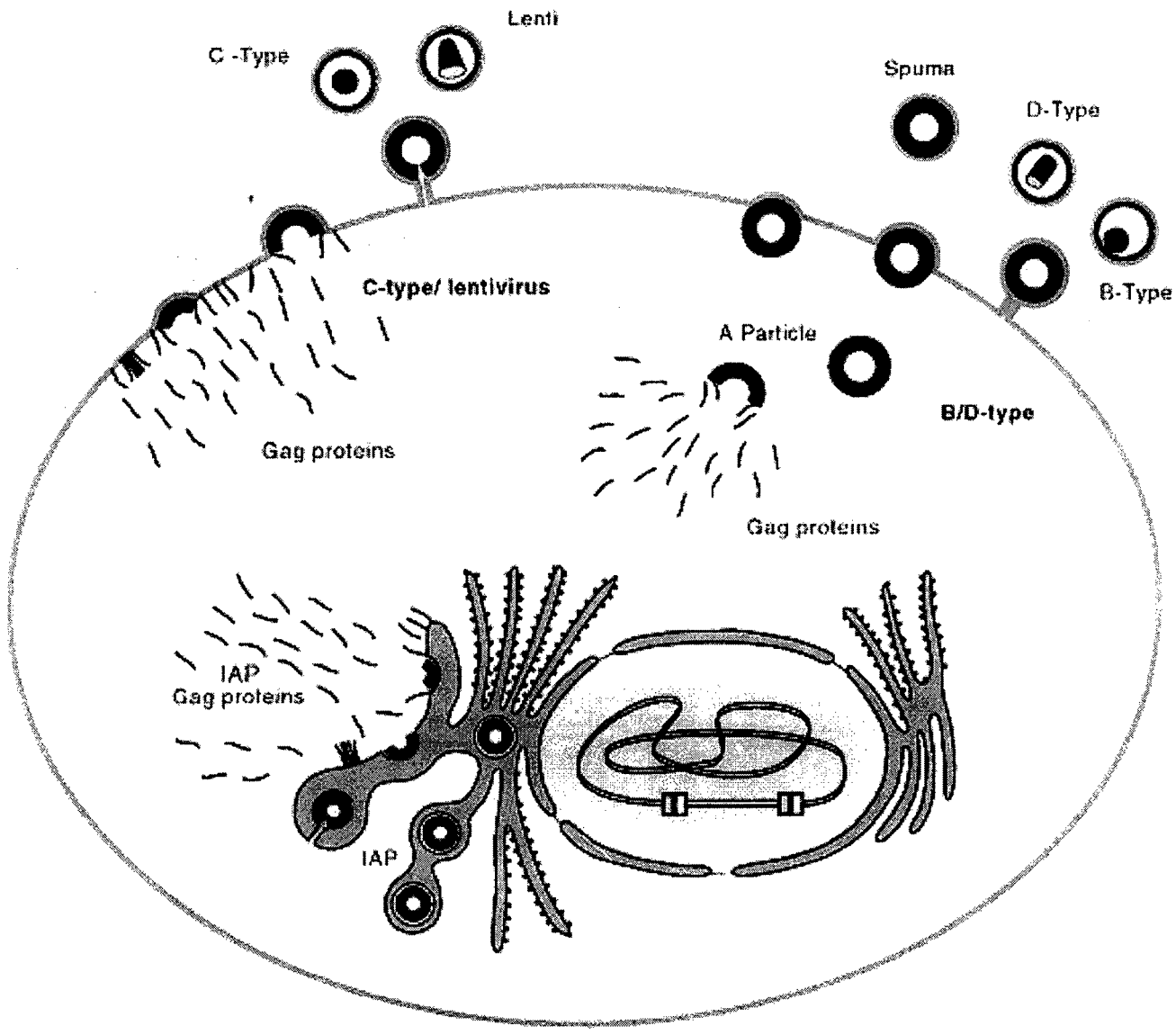
Type B/D particles are enveloped, extracellular particles containing an electron dense core characterized by immature capsids. These particles first assemble within the cytoplasm and are then transported to the plasma membrane for budding, and they contain glycoproteins projections on their envelope. MMTV viruses are an example of type B viruses. Type D particles are intracellular particles containing an acentric mature core with less prominent surface glycoprotein projections. Mason-Pfizer monkey virus (M-PMV) is an example of type D virus.

Type C virus form incomplete intracellular particles containing electron dense crescent shaped cores at membranes of infected cells. These particles initiate assembly of capsid at the inner face of the plasma membrane, and acquire surface glycoprotein projections on their envelope during budding from the host cell membranes. All Lentiviruses and type C virus particles, including HIV-1 and RSV/ MuLV, respectively, possess a type C morphology. In spite of these differences in the cellular location of capsid formation and budding, it is likely that the different viral types share many common mechanisms of assembly. It has been shown that altering the genetic composition or cellular conditions of a virus can allow a virus to switch its assembly mode from one pattern to another (122, 149, 194, 451, 461, 474). For instance, it has been observed that a single amino acid change in the matrix protein of the type B M-PMV causes it to assemble at the plasma membrane rather than in the cytoplasm (461). On the other hand, deletions in HIV-1 matrix can cause budding of cytoplasmic virions into endoplasmic reticulum, similar to the type A particle pattern (149), or assembly of

capsid at a perinuclear location, like B/D type particle pattern (519).

Figure 1.7 Retroviral patterns of assembly.

Retroviral assembly can be divided into three main classes: Types A, B/D, and C. Type A ('intracisternal particles') are non-enveloped immature virus particles that are only found inside cells, and represent the assembly pattern of endogenous retroviruses. Type B/D are viruses that assemble their capsids in the cytoplasm and later bud out through the cell membrane acquiring their envelope from the hosts membrane. An example of this group includes MMTV (B type) and M-PMV (D-type) viruses, which differ in the location and shape of the cores in the viruses. Type C/lentiviruses are viruses that assemble their capsids at the plasma or endosomal membranes, acquiring their membranes from the host at the same time. An example of this group includes ALV and HIV-1.


Lenti
C -Type
Spuma
D-Type
C-type/ lentivirus
B-Type
A Particle
B/D-type
Gag proteins
Gag proteins
IAP
Gag proteins
IAP

1.4.2 Role of Precursor Proteins and RNA in HIV-1 Assembly.

HIV-1 assembles into morphologically distinctive non-infectious immature and infectious mature virions. The virus primarily assembles and buds from cells as a noninfectious, immature, spherical particle that is organized by a layer of Gag proteins that are associated with the inner viral membrane (Figure 1.1). Upon budding, significant rearrangement occurs in the immature particles to form mature, infectious virions. The mature capsids are mostly conical. Following Gag processing, 1500 copies of the mature CA protein reassemble to form the mature capsid (Figure 1.1) (42, 43, 186, 336). Recent studies imply that the immature core dissociates upon PR cleavage and the mature core reassembles from liberated CA, rather than the immature core condensing into the mature core (41, 42).

The distinct morphologies of the immature Gag lattice and the capsid lattice presumably reflect specific requirements for organizing viral assembly in the producer cell and coordinating viral replication in the new host cell (164, 203, 565). The viral 'core' constitutes the capsid and its contents and it is released into the cytoplasm of a newly infected cell to initiate a new cycle of viral replication (3).

Although Gag itself encodes all the necessary information for retroviral particle assembly, the assembly requires nonspecific RNA interactions both *in vivo* and *in vitro*, and is assisted by host factors *in vivo*, including trafficking factors, assembly chaperones, and the ESCRT budding pathway (30, 303, 458, 564).

1.4.2.1 Gag Assembly

Gag alone is sufficient for the formation and release of extracellular Gag viral-like particles (VLPs) (126, 164, 525, 565). Gag molecules can assemble into spherical, immature VLPs *in vitro* (56, 57, 211). Gag is synthesized and transported to the plasma membrane. Gag-Gag interactions direct the assembly of virus particles that eventually bud off from the plasma membrane. Mutational analysis studies first performed with RSV Gag showed that there are three functional assembly domains found within retroviral Gag precursor: M, I, and L domains (565). It is these domains that facilitate, respectively, binding of Gag to membranes, Gag-Gag multimerization, and VLP release. The M domain is the membrane-binding domain and is comprised of the 31 N-terminal residues of MA. The I domain is defined as the region contributing to the tight packing of Gag molecules during viral assembly, and it is located within the N-terminus of NC (24, 565, 592). The L domain is in p6 sequences within Gag, and it is required to facilitate budding (204). Even though these three domains represent distinct assembly domains within retroviral Gag polyproteins, additional assembly functions within HIV-1 Gag are situated outside of these domains. For instance, the Gag interaction is mediated largely by a region spanning the C terminal domain of the CA (2, 7, 38, 365, 453, 571) and adjacent SP1 spacer, in addition to the N terminus of the NC domain where the I domain is located (2, 84, 132, 310, 333, 393, 484, 587). It has been shown that SP1 is crucial for the generation of virus particles, and is required for a higher ordered Gag multimerization (310, 393). Another example is shown by the HIV-1 major homology

region (MHR) within the C terminal domain of CA, where point mutations within this region result in severe defects in assembly (365). Similarly, CA mutations positioned C terminal to the MHR have resulted in elimination or severe reduction of particle assembly (132, 282, 365, 542, 588). Collectively, these results suggest that the M, I, and L domains be considered as conserved functional retroviral assembly domains but do not include all the necessary regions within HIV-1 Gag concerned with assembly.

1.4.2.2 Site and Stages of Gag Assembly

In many cell types, the assembly of HIV-1 occurs at the inner surface of the cell plasma membrane (164, 190, 241, 415, 418, 565, 577). Therefore, the anchorage of Gag precursors to the cytoplasmic surface of the plasma membrane may play a role in concentrating and aligning the Gag precursors to allow them to interact with each other.

However, the subcellular localization of HIV-1 assembly is a subject of controversy. CD4+ T cells and macrophages are the major targets of HIV-1 infection *in vivo*; however, the site at which HIV-1 assembles and buds differs between these two cells. In CD4+ T cells such as HEK 293T, Hela, and T cell lines, it is commonly accepted that HIV-1 buds from the plasma membrane (162, 191, 197, 244, 277, 383, 384, 395, 405, 420), although this has been recently disputed (130, 207, 436, 499, 543). On the other hand, the main site of viral assembly in macrophages has been proposed to be intracellular vesicles potentially corresponding to multivesicular bodies (MVB), where virus may remain infectious for subsequent exocytosis (309, 372, 403, 408, 435, 450), but this too has been lately contested (156, 229, 281, 400, 475, 557).

To address these controversies, a recent study using a subcellular fractionation method that separates the plasma membrane from late endosomal MVB was combined

with pulse-chase labeling of Gag to analyze Gag trafficking in HEK 293T infected cells. It was found that the majority of newly synthesized Gag is primarily targeted to the plasma membrane and released efficiently, however, a significant fraction of the remaining cell surface associated Gag was subsequently internalized to the MVB, where it accumulated intracellularly (156). Furthermore, in another recent study, the site of Gag localization and virus assembly in primary human macrophages was examined by using biarsenical dyes that become fluorescent when they bind a small target sequence introduced into HIV-1 Gag. It was observed that Gag localization occurred both at the plasma membrane and in MVBs (206).

A study attempting to clarify the path of Gag trafficking examined traffic of Gag in several cell types, including Hela cells, Jurkat cells, and T cells. They used two techniques, biarsenical/tetracysteine (TC) labeling and release from a cycloheximide block, to follow the trafficking of newly synthesized HIV-1 Gag by live cell imaging. Gag first appears diffusely distributed in the cytosol, accumulates in perinuclear clusters, passes transiently through a multivesicular body MVB, and then travels to the plasma membrane (PM). These findings favor a model in which transient Gag localization to MVBs compartments is a trafficking mechanism common to all cell types (436).

1.4.2.3 Role of MA in Targeting Gag to Membrane

In many retroviruses, the Gag precursor protein attaches to the lipid membrane via a myristic acid moiety added post-translationally to the amino terminus of the MA sequences (47, 205, 239, 456, 462). However, this attachment is weak and is not sufficient for membrane attachment (462, 565), but is further strengthened through ionic

interactions between the phospholipids in the membrane and a sequence of basic amino acids residues within the MA sequences (109, 459, 460, 566, 592). These ionic interactions are supported by the fact that some retroviral MA proteins lack myristoyl modifications, and membrane binding is mediated exclusively by ionic interactions in such cases (110, 144).

Studies have elucidated that MA domain targets Gag to the plasma membrane and triggers particle assembly at the appropriate place and time (458). Membrane binding of HIV-1 Gag is mediated by two signals in MA: the N-terminal myristic acid and the conserved basic patch region within MA (47, 205, 592). MA is cotranslationally myristoylated and serves to anchor Gag to the membrane (243, 376, 478).

In addition, deletions in HIV-1 MA caused defects in virus production by inducing intracellular accumulation of Gag or redirection of VLP assembly to intracellular locations (60, 171, 180, 454, 547, 583). Experiments using MA mutants with amino acids changes in the highly basic domain (amino acids 17-31) (47, 205, 592) and between residues 84 and 88 have proved that the highly basic domain of MA contains a major determinant of HIV-1 Gag plasma membrane targeting, and that mutations between residues 84 and 88 disrupted plasma membrane targeting through an effect on the basic domain (418). In addition to membrane targeting, MA has been implicated in the binding of Gag to membrane that is mediated through its "M" domain (429). Both the covalent binding of myristate to the N-terminal Gly residue of MA post-translationaly and the highly basic region of MA, are essential for Gag binding to membrane and hence required for virus assembly (47, 194, 205, 243, 376, 428, 592). Myristate moieties embed into the lipid bilayer by hydrophobic interactions. Since that

intact Gag binds more tightly to membranes than the isolated MA protein, which led to the suggestion that binding may be mediated by a myristyl switch mechanism (382). Therefore, the myristate moiety is considered; to be regulated by a mechanism termed a myristoyl switch (240, 414, 427, 457, 477, 478, 511, 527, 593). The current model suggests that the sequestering of the N-terminal myristate residues in the MA protein and a structural change then exposes the myristate and enhances Gag membrane binding. According to the myristyl switch model, the degree of exposure of this fatty acid is important for Gag membrane binding, and while the myristate moiety is highly exposed in the context of Gag, it is sequestered within MA following proteolytic cleavage of the precursor by viral PR (240, 427, 511, 527, 593). Gag mutations at sites distinct from the M domain could cause impairment of membrane association through conformational changes of the N-terminal structure of Gag (340).

The basic amino acids in the highly basic domain of MA form a positively charged surface that further facilitate Gag binding to membrane by promoting an electrostatic interaction with acidic phospholipids, termed phosphatidylinositol (4,5) bisphosphate, PI(4,5)P2, that is concentrated in the inner leaflet of the membrane (82, 109, 243, 376, 413, 592). *In vivo* studies have shown that upon depletion of cellular PI(4,5)P2, HIV-1 Gag was incorrectly directed to internal membranes suggesting that it is required for proper membrane targeting (413). Therefore, PI(4,5)P2 appears to be involved in membrane targeting and budding *in vivo* (413), and binding to the PI(4,5)P2 probably favors the myristoyl exposure (478). Cholesterol also plays an important role in Gag targeting and particle assembly (417), but the molecular mechanism by which Gag senses cholesterol is unclear.

It is these interactions, together with myristate, that stabilize the association of the Gag protein with the membrane (298, 434, 583, 592). However, a minimal 16 KDa Gag construct lacking matrix sequences has been shown to form VLPs *in vivo*, and is composed of an N-terminal myristylation signal, the C-terminal half of CA, SP1, a leucine zipper domain of yeast GCN4 replacing NC, and a PPPY motif replacing p6 (2).

1.4.2.4 Specific Membrane Domains Involved in Gag Assembly

It has been suggested that after the myristate moiety has inserted into the membrane, it drives the Gag proteins to specific membrane domains where an electron-dense, crescent shaped virion complex forms on the cytoplasmic side of the plasma membrane (59, 538, 575). It has been reported that HIV-1 proteins produced in infected T cells are associated with specific membrane domains called lipid rafts (405). Lipid rafts are enriched in cholesterol, sphingolipids, glycosylphosphatidylinositol (GPI)-linked proteins such as Thy-1 and CD59, ganglioside GM1, Src family kinases Lck and Fyn, and caveolin (12, 85, 245, 486, 508). They possess specific chemical and physical properties allowing them to be separated from non-lipid raft membrane, such as resistance to extraction with cold, non-ionic detergents such as Triton-X 100 and higher boyant density (44, 349). Although that CD45 is highly expressed on cells surfaces, however, it is excluded from lipid raft domains, and it is poorly incorporated into viral particles. It was also determined by confocal fluorescence microscopy that lipid raft-associated molecules co-localize with HIV-1 proteins on the cell surface (405) suggesting that HIV-1 buds preferentially from those domains. In addition, numerous reports have indicated that Gag is targeted to lipid raft microdomains of the plasma

membrane (345, 406, 416). Most of these studies assigned Gag to lipid rafts since density gradient analyses indicated that a significant fraction of Gag displayed buoyant density in cold triton-X 100 that is similar to that of lipid rafts. Another line of evidence that supported the rafts playing a role in HIV-1 assembly was the use of cholesterol-depleting agents. These agents cause alterations in raft structures and were reported to decrease the release of HIV-1 particles from infected cells (416) as well as to reduce the infectivity of released particles (416, 591). Interestingly, cholesterol-lowering statins were shown to inhibit HIV-1 infection and lower viral loads in patients (121).

In addition to lipid raft domains, other studies have indicated that the tetraspanin-enriched microdomains (TEMs) in plasma membranes of T cells and Hela cells may also involved in HIV-1 budding (37, 276, 297, 409). Both lipid rafts and TEMs are enriched in cholesterol; however, they are distinct regions because lipid rafts are not enriched in tetraspanins (160, 238, 422), and may represent multiple sites for viral budding.

1.4.2.5 Mechanism of Budding of Gag VLPs

Multimerization between Gag molecules at the membrane leads to the formation of electron dense patches, giving rise to budding particles that eventually pinch off from the plasma membrane. Mutational analyses demonstrated that deletion of the p6 region from the C-terminal of Gag, produced a striking defect in virus particles production (204, 253). Instead of budding off from the plasma membrane, mutant virions remained tethered to the cell surface. Mutations in a highly conserved PTAP motif within p6 yielded the same phenotype (123, 253). Collectively, these results identified p6, and specifically the PTAP motif within p6, as playing an essential role in HIV-1 budding.

In the past several years, many studies have uncovered the identity of host proteins that play a role during the budding of virions from the plasma membrane. Endosomal sorting complex required for transport (ESCRT) family Members of the ESCRT pathway have been established to be important for viral budding (30, 166, 374, 395). Normally this pathway is involved in sorting ubiquitinated cytoplasmic proteins into the multivesicular body (MVB) pathway that leads to lysosomal degradation or exocytotic release from the cell (523). Within this pathway, several host proteins are incorporated into HIV-1 and assist in efficient viral release including: Tsg101 (523); Alix (AIP1) (521, 543); Vsp28 (523); ubiquitin (423); Vsp4a (543); and Tal (10).

Tsg101, the most notable of these proteins, and its yeast ortholog Vps23, are components of a 320 KDa complex, ESCRT-I, that is essential for the sorting of ubiquitinated proteins into MVB and for endosomal targeting in yeast and mammalian cells (15, 33, 291). Tsg101 is recruited from internal sites to the plasma membrane by Gag (373). It has been shown that Gag associated with Tsg101 is ubiquitinated (Ub) (398) and this ubiquitination is thought to promote the Gag and Tsg101 interaction (167). The ′budding′ and membrane fusion events that lead to the formation of a vesicle within the MVB are equivalent to the extracellular budding of enveloped viral particles differing only in the contents of the ′vesicle′ and in the cellular location at which they occur. A proposed hypothesis is that viral proteins recruit the machinery that normally mediates MVB formation to sites of virus budding at the plasma membrane. One way for Gag to do that is by binding to Tsg101, which is thought to recruit additional cellular factors (e.g. Vps28) to the site of budding (166). One study supported this hypothesis by reporting that HIV-1 particle budding is highly dependent on the integrity of the Tsg101

C-terminal domain (which contains a binding site for Vps28) and suggested that an intact ESCRT-1 complex is crucial for PTAP-type L-domain function (374). In addition, Both the PTAP within p6 region of Gag and Ub-binding domains at the N-terminal of Tsg101 recruit the cellular machinery required for budding, and are required for efficient viral particle release from the plasma membrane (198).

1.4.3 Role of RNA in HIV-1 Assembly

Viral RNA and Gag proteins are the main structural components that act as a scaffold where all other components of the retroviral particle assemble. NC is the major RNA binding domain of Gag and is involved in two types of RNA binding: The two zinc binding motifs in NC appear to be involved with binding to stem loop structures in specific RNAs, while the regions flanking the first zinc finger that are rich in basic amino acids appear to be involved in non-specific ionic binding to RNA (84). Intact zinc fingers, essential for the specific incorporation of HIV-1 genomic RNA into virions, have been shown to recognize the Ψ packaging signal in the HIV-1 RNA leader sequence (111). Mutations in the basic amino acids regions will also decrease genomic RNA packaging, but probably through reducing the non-specific binding component of NC to the viral RNA. NMR studies have shown that the HIV-1 NC interaction with stem loop 3 (SL3) in the Ψ RNA recognition element involved both the first zinc finger and the basic amino acid regions flanking the first zinc finger (118). Other studies have shown that the basic residues flanking the first zinc finger appear to be identified with the interaction domain (I domain) in avian and mammalian retroviruses (24, 40, 556). These residues are involved in Gag-RNA (57, 61) and Gag-Gag interactions (484) and contribute to the tight packing of Gag molecules during viral assembly (24, 556). The

basic amino acid residues have a large effect on Gag-Gag interactions, but only a minimal effect on the density of any mutant virions that are still produced (83). The mechanism by which the I domain (basic residues) functions during viral assembly could reflect its ability to bind Gag to RNA, thus facilitating later direct protein/protein interactions between Gag molecules. NC sequences might also directly participate in Gag-Gag interactions, and cross-linking studies indicate a close interaction between NC sequences in adjacent Gag molecules in HIV-1 (381). It has been noted that while deletions of NC in HIV-1 reduce assembly and production of virus ten fold, replacement of NC with protein sequences known to facilitate interprotein contacts, but with poor RNA binding ability, can return the production of these virus to wild type levels (588). Since other evidence indicates an important role of NC in Gag-RNA binding, the ability of these chimeric NC-deleted Gag molecules to produce viral particles may have resulted from membrane replacing RNA as a scaffold for Gag assembly.

RNA has been proposed to act as a scaffold for aligning Gag molecules and facilitating their interaction with each other (27, 54, 84, 295). *In vitro* studies using truncated Gag molecules have showed that RNA is important in facilitating a membrane-independent interaction between Gag molecules, and that this RNA need not to be viral. For instance, in *in vitro* assembly studies using *Eschericia coli (E.coli)*-produced RSV or HIV-1 peptides containing only capsid-nucleocapsid (CA-NC) sequences, it was found that these peptides only assembled into hollow cylinders in the presence of added RNA, with the cylinder length dependent upon the length of the RNA (58). The RNA used in this study was isolated from *E.coli*, suggesting that assembly did not depend upon the presence of viral specific RNA. In another study (209), similar cylindrical structures

were also reported to form *in vitro* in the absence of RNA when HIV-1 CA was used alone, but RNA greatly facilitated the speed of the reaction. On the other hand, mutations in the basic residues impaired NC's nonspecific RNA-binding activity. This was shown by immobilizing NC mutants on glutathione-agarose (GST) beads and incubating them with radiolabeled RNA. Compared to that of wild type GST-NC, a decrease in RNA-binding was observed with the NC mutants as an increasing number of basic residues were substituted with alanine (84). In addition, RNase A was shown to impair Gag-Gag interactions whether *in vitro* (54) or *in vivo* (295).

Most mutations in the zinc fingers, while affecting selective genomic RNA incorporation, do not inhibit the packaging of cellular RNA into virions, and do not affect viral assembly or viral density (40, 202). Furthermore, it was reported that RNA plays a structural role in retrovirus particles. RNase treatment of viral core preparations disrupts the cores almost entirely, and in the absence of an mRNA species with a specific packaging signal, cellular RNAs are incorporated into the virion (397). These data imply that RNA in the cytoplasm, viral or non-viral, may serve as a scaffold to bind and concentrate inter-protein interactions.

1.4.4 GagPol Assembly

During the viral assembly process, GagPol is incorporated into Gag particles by interacting with Gag (430, 505, 506, 513). The *in vivo* interaction of GagPol with Gag has been less well documented than the interaction between Gag molecules. A cytoplasmic interaction between Gag and GagPol, independent of myristylation of either precursor, has been reported (327). However, while the incorporation of GagPol into extracellular Gag particles does not depend upon myristylation of GagPol, it does

depend upon myristylation of Gag (430, 506), suggesting that the GagPol incorporation does not depend on its own insertion into the plasma membrane. In addition, it has been shown that, in the absence of Gag, GagPol accumulates in the cytoplasm and is not detected at the plasma membrane in the absence of Gag (222).

Studies have shown that GagPol interacts with Gag through intermolecular interactions, between homologous Gag sequences in both molecules (430, 505, 506, 513). The Gag/GagPol interaction appears to be dependent upon CA sequences. Deletions of the highly conserved major homology region (MHR) in CA sequences within either Gag or GagPol significantly impaired GagPol incorporation into Gag VLPs (506). In another study it has been shown using a chimeric HIV-1/MLV GagPol expression system, that both HIV-1 CA MHR and adjacent CA C-terminal sequences are required for efficient GagPol incorporation into virus particles (252). In this study, MA and NC sequences were not found to be important for GagPol incorporation. In contrast, different results were reported by another group stating that substitution mutations in the CA MHR of GagPol have no detectable effects on GagPol incorporation into Gag particles (365). These discrepancies between these two studies could be due to the different effects of small mutations compared to larger deletions and replacements.

The interaction of GagPol with Gag requires RNA. However, putting RNA-binding mutations into either Gag or GagPol indicated that the requirement for RNA reflects a requirement for an RNA-facilitated Gag multimerization, and not a direct interaction of GagPol with RNA (295).

In addition, another group has demonstrated that the MLV Pol domain can be incorporated into virus particles, suggesting that the Gag domain in MLV GagPol is not

absolutely required for its assembly into particles (48). This observation suggested that interactions could occur directly between the Pol and Gag in the Gag/GagPol complex, which is supported by the Pol/Gag interaction occurring naturally in another retrovirus, the human foamy virus (HFV), where Pol and Gag are made from separate genes (516). In HIV-1, it was also reported that coding sequences upstream of the RT domain within GagPol are not essential for Pol incorporation into Gag VLPs (78), and we have also shown that Pol is incorporated into Gag VLPs via RT sequences interacting with Gag p6 (72). In that report, we also demonstrated that Pol alone is sufficient for facilitating the select packaging of $tRNA^{Lys}$ isoacceptors into Gag VLPs. We (Chapter 2) and others (342) have also demonstrated that viral RT expressed *in vivo* is also incorporated into Gag VLPs suggesting that RT sequences in Pol interact with Gag. Liao *et al* have mapped the interacting domains between RT and Gag to include MA and p6 sequences within Gag and the thumb domain in RT (342). Further discussion of the role of RT sequences to facilitate interactions within the $tRNA^{Lys}$ packaging/annealing complex will be presented in chapter 2.

Because the membrane surrounding HIV-1 is enriched in lipid rafts, it is hypothesized that HIV-1 assembles at those regions of cell membrane enriched in lipid rafts. Examination of the cellular distribution of newly-synthesized Gag and GagPol revealed that while only 15% of Gag was found associated with lipid rafts, >95% of newly-synthesized GagPol was associated with lipid rafts (222). Since GagPol does not associate with membrane independently of Gag, it appears that a Gag/GagPol complex is formed at the membrane composed of all newly synthesized GagPol, but only15% of newly synthesized Gag. It can be calculated that the ratio of Gag:GagPol in this lipid

raft-associated complex is far lower than that found in the immature virion, and the implications of these observations suggest a multistage assembly model for HIV-1. This is discussed in more detail in the discussion section of chapter 2.

1.5 The Primer tRNA's for Reverse Transcription

Different retrovirus family members use different cellular tRNA molecules as primer for reverse transcription. The different primer tRNAs used by some retroviruses are listed in Table 1.1. In avian retroviruses, such as avian sarcoma virus (ASV) and avian leucosis virus (ALV), $tRNA^{Trp}$ is the primer (150, 226, 440, 489, 549, 550). Whereas, most murine retroviruses such as Moloney murine leukemia virus (Mo-MuLV) use $tRNA^{pro}$ (225, 438, 528). However, murine retroviruses have also been reported to use other tRNAs primers (95). Three main $tRNA^{Lys}$ isoacceptors exist in mammalian cells (449). $tRNA^{Lys1,2}$ is the primer used by several retroviruses such as M-PMV and HFV (328). However, $tRNA^{Lys3}$ is the primer used by mouse mammary tumor virus (MMTV) (439), as well as for many lentiviruses including equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), HIV-1, and HIV-2 (328, 360).

Although their nucleotide sequences vary, however, all tRNA species are heavily modified, and form the characteristic L-shaped three-dimensional tRNA structure. It has been reported that some of these modified bases are essential for retroviral reverse transcription, because reverse transcription initiation from *in vitro* synthesized unmodified tRNA primers is less efficient (17, 18, 264-266, 388, 487, 569).

Table 1.1 Different tRNA primers that are utilized by different retroviruses.

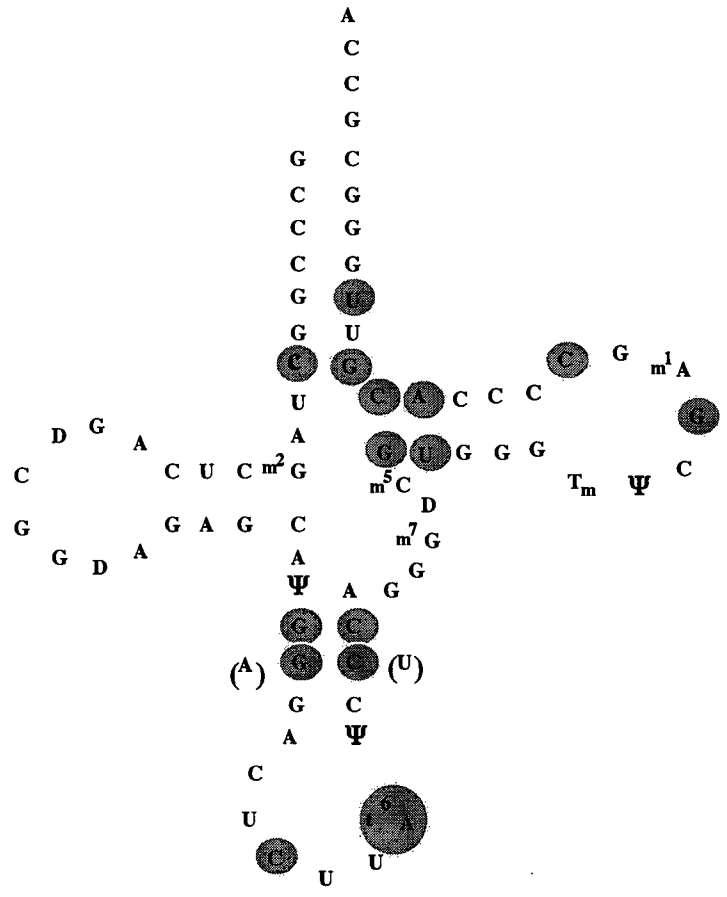
Examples of the different tRNA primers that are used by different retroviruses.

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	$tRNA^{Trp}$

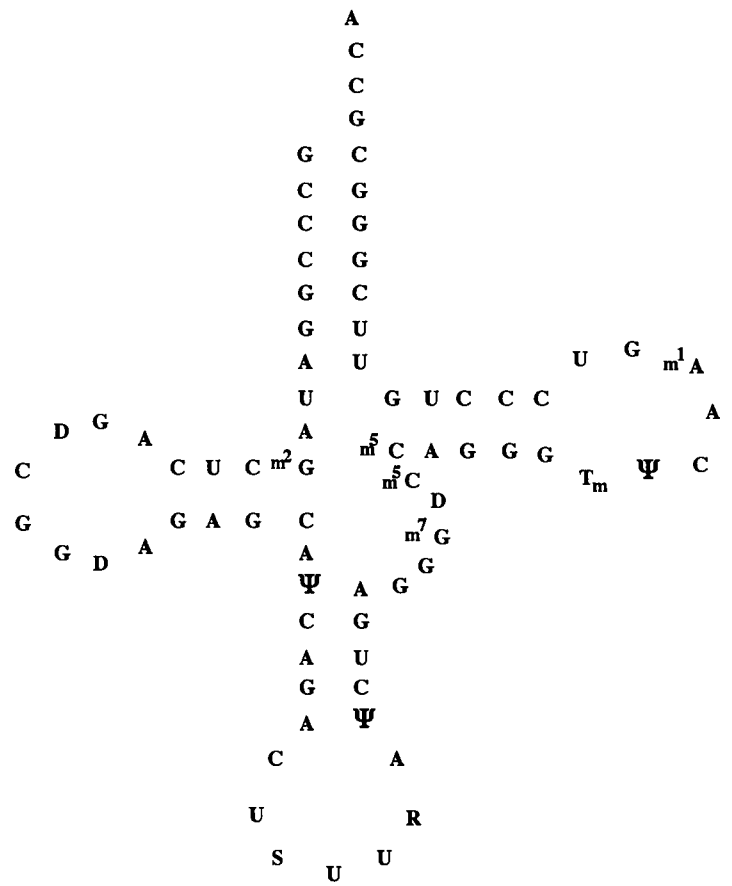
1.5.1 Selective Packaging of Primer tRNAs in Retroviruses

The term "selective packaging of tRNA" refers to the increase in the percentage of the low molecular weight RNA population, representing primer tRNA in virions, in going from the cytoplasm to the virus. This represents an enrichment of tRNA species within the virus. For instance, in avian myeloblastosis virus (AMV), the relative concentration of $tRNA^{Trp}$ changes from 1.4% to 32% (549). In lentiviruses, including HIV-1, $tRNA^{Lys3}$ serves as the primer tRNA (328, 360). In HIV-1 produced from COS7 cells transfected with HIV-1 proviral DNA, both primer $tRNA^{Lys3}$ and the other major $tRNA^{Lys}$ isoacceptors, $tRNA^{Lys1}$ and $tRNA^{Lys2}$, are selectively packaged. $tRNA^{Lys1,2}$,which differs from $tRNA^{Lys3}$ by 14 or 16 bases, represents two $tRNA^{Lys}$ isoacceptors differing by one base pair in the anticodon stem (Figure 1.8). The function of $tRNA^{Lys1,2}$ in HIV-1 is not known . The $tRNA^{Lys3}$: $tRNA^{Lys1,2}$ ratio in the virion reflects the cytoplasmic ratio, even when the cytoplasmic ratio is altered (257). The relative concentration of $tRNA^{Lys}$ changes from 5-6% in the cytoplasm to 50-60% in the virus (358). HIV-1 contains a reported number of 20 molecules of $tRNA^{Lys}$/virion. It has been shown that HIV-1 particle may contain an estimated 12 $tRNA^{Lys1,2}$ molecules and 8 $tRNA^{Lys3}$ molecules as determined using hybridization probes specific for each $tRNA^{Lys}$ isoacceptor (257, 358, 360). The selective packaging of primer $tRNA^{Lys3}$ is required for optimizing infectivity of the HIV-1 population (174), and an understanding of this process requires an understanding of how viral RNA, $tRNA^{Lys}$, and viral and cellular proteins interact during HIV-1 assembly.

Figure 1.8 Sequences of $tRNA^{Lys1,2}$ and $tRNA^{Lys3}$. $tRNA^{Lys3}$ is the primer for reverse transcription in HIV-1. $tRNA^{Lys1,2}$ is composed of two different $tRNA^{Lys}$ sequences differing in only one base pair in the anticodon stem in parenthesis. The circled bases in $tRNA^{Lys1,2}$ show those positions where nucleotide identity differs from that in $tRNA^{Lys3}$.



tRNA^{Lys1,2}



tRNA^{Lys3}

1.5.2 The $tRNA^{Lys}$ Packaging/Annealing Complex

We have defined a $tRNA^{Lys}$ packaging/annealing complex, which includes $tRNA^{Lys3}$, human lysyl-tRNA synthetase (LysRS), Gag, GagPol, and viral genomic RNA (Chapter 2). Figure 1.10 depicts a proposed model for the formation process of the $tRNA^{Lys}$ packaging/annealing complex. We have proposed a scenario in which a Gag/GagPol/viral RNA complex interacts with a $tRNA^{Lys3}$/LysRS complex, with Gag interacting specifically with both GagPol and LysRS, and GagPol also interacting with $tRNA^{Lys}$. The facts supporting this model are described in detail throughout the thesis, and are reviewed in both the discussion section of Chapter 2, and the general discussion. Some of the components of this complex have been reviewed previously in this thesis; however, a detailed description of other components will be reviewed next.

1.5.3 Viral Proteins Regulating $tRNA^{Lys}$ Packaging into HIV-1

1.5.3.1 Reverse Transcriptase (RT) Sequences

In HIV-1, the selective packaging of $tRNA^{Lys}$ isoacceptors requires the presence of both Gag and GagPol, but occurs independently of the processing of these proteins and genomic RNA packaging (275, 358). Studies have shown that Gag VLPs do not package $tRNA^{Lys}$ unless GagPol is also incorporated, and that RT sequences in GagPol are required for this process (296, 358). Studies of RT-deficient viruses have shown that these viruses contain a random subset of cellular tRNAs indicating the essential role of RT enzyme in tRNA selective packaging, (116, 341, 359, 425, 440, 488), although since

tRNA^{Lys} incorporation is independent of protease-processing, the RT sequences are believed to function within the GagPol precursor (341, 358).

The HIV-1 RT domain has been resolved into several structural subdomains termed fingers, palm, thumb, with a connection subdomain bridging the other subdomains to the C-terminal RNase H subdomain (Figure 1.5)(271). *In vivo* studies have shown that RT thumb within GagPol plays an important role in HIV-1 tRNA^{Lys} packaging. This work has shown that C-terminal deletions of sequences within GagPol did not inhibit HIV-1 tRNA^{Lys} incorporation until deletion of the thumb domain (296). *In vitro* studies have also shown that RT thumb subdomain is essential for the interaction of RT with tRNA^{Lys3} and have supported the above-mentioned *in vivo* study. *In vitro* cross-linking studies determined that the RT peptide bound to tRNA^{Lys3} contained thumb sequences (134) (388).

1.5.3.2 Nucleocapsid (NC) Sequence

As discussed above, NC has been implicated to have crucial roles in many steps in HIV-1 replication cycle, including tRNA^{Lys3} annealing (115, 216). There is no evidence that NC has a direct role in tRNA^{Lys} packaging into HIV-1 (255).

1.5.4 Cellular Proteins Regulating tRNA^{Lys} Packaging into HIV-1: The Role of Lysyl-tRNA Synthetase (LysRS).

1.5.4.1 Aminoacyl-tRNA Synthetases (aaRS) Classification

LysRS is an important member of the aminoacyl-tRNA synthetases (aaRS) family. The aaRSs are a family of enzymes well known for their role in protein synthesis. These essential proteins are found in all forms of life and are responsible for

charging by covalently attaching their cognate tRNAs with the correct amino acid in a high fidelity reaction. During protein synthesis and upon leaving the ribosomes, tRNA must reassociate with their cognate aaRS to regenerate the aminoacyl-tRNA required during the reaction. This regeneration process occurs in a two-step reaction in order to esterify amino acids to the 3′ end of their cognate tRNA substrate. Therefore, aaRSs catalyze a two-step reaction that is responsible for pairing a specific tRNA to a specific amino acid.

In the first step they activate their amino acid by forming an aminoacyl-adenylate (aa-AMP) intermediate, in which the carboxyl of the amino acid is linked in to the alpha-phosphate of ATP, by displacing pyrophosphate. And then they attach the correct tRNA molecule; the aminoacyl group of the aminoacyl-adenylate is transferred to the 2' or 3' terminal OH of the conserved CCA-3′ end of the cognate tRNA depending on the synthetase class (29).

This classic family of enzymes is actually capable of a broad range of functions that not only impact protein synthesis, but also extend to a number of other critical cellular activities including playing roles in amino acid biosynthesis, transcriptional and translational regulation, DNA replication, tRNA processing, RNA splicing, RNA trafficking, apoptosis and aspects of eukaryotic cell biology related to cytokine function and cell control (375, 576).

aaRSs are classified in two different groups: class I and class II based on their sequence alignment and structural features. Table 1.2 lists examples of the different classes of aaRSs. Each class is further divided into three subclasses a, b and c based on the chemical characteristics of the amino acids substrate (263). Mammalian LysRS is a

class IIB synthetase. There are many differences that exist between the two classes. Class I synthetases commonly exist as monomers while class II exist as dimers or tetramers. Class I synthetases are also different in that they are larger, more specific, and more hydrophobic than most class II synthetases. Class I synthetases recognize the anticodon in order to aminoacylate their related tRNAs, whereas class II synthetases have no interrelation with their constrained tRNA's anticodon at all. Furthermore, class I synthetases attach the carboxyl group of their target amino acid to the 2' OH of adenosine 76 in the tRNA molecule, while class II synthetases attach their amino acid to the 3' OH of their tRNA, except for phenylalaninyl-tRNA synthetase which uses the 2' OH (526).

Eukaryotic pre-tRNAs are synthesized in the nucleus and need extensive processing (removal of 5′ and 3′ termini and introns, various base modifications, and post-translational addition of CCA to the 3′ terminus) to yield functional tRNAs (248, 353, 570). A high molecular weight aaRS complex has been found in the nucleus, and some nuclear tRNAs are aminoacylated in the nucleus before export to the cytoplasm in what is believed to be a proofreading mechanism (353, 493). Eukaryotic tRNA synthetases also exist in the mitochondria where protein synthesis also occurs. Some mitochondrial aaRSs are coded by the mitochondrial genome, such as human histidyl-tRNA synthetase (410), while others are encoded by the nuclear genome. For example, both cytoplasmic and mitochondrial forms of LysRS are coded by the same gene, and are produced by alternate splicing of the same primary transcript (534).

Table 1.2 Table outlining the two classes of tRNA synthetases

Schematic table outlining the two classes of tRNA synthetases. AaRS are further divided structurally into classes Ia, Ib, and Ic and IIa, IIb, and IIc. The eukaryotic nine synthetases and the three auxiliary proteins that are involved in the multisynthetase complex are also indicated in bold.

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

1.5.4.2 LysRS Structure and Properties

Human LysRS is a tRNA binding protein that is responsible for aminoacylating the $tRNA^{Lys}$, and it belongs to the subgroup (class IIb) within the aaRS family with the closely related aspartyl-tRNA synthetase (AspRS) and asparginyl-tRNA synthetase (AsnRS)(Table 1.2)(145, 146). LysRS exists as a homodimer with an apparent molecular weight mass of 68 KDa. LysRS like other class IIB aaRSs is characterized by an anti-parallel β-sheet active site, and it recognizes the anti-codon within the tRNA. The crystal structure of the tetrameric form of human LysRS has recently been reported (219).

In eukaryotic cells, LysRS is found as part of a high molecular weight aminoacyl tRNA synthetase complex (HMW aaRS). The molecular mass of this complex is 1.5 KDa, and it includes eight other aaRSs (MetRS, GluRS, ProRS, IleRS, LeuRS, AspRs, GlnRS, ArgRs), and three auxiliary non-aaRS proteins (p38, p43, p18) (576). To date, the function of this cytoplasmic HMW aaRS complex is still to be determined. p38 is a crucial component for the assembly of this complex and acts as a scaffold protein on which other components are assembled within the complex (299). It has been shown that LysRS, ArgRS, and p43 are strongly and directly associated with p38 to form a core to which other components are then added (447, 467). p42 protein is located within the center of the complex (407) and is associated with arginyl-tRNA synthetase via its N-terminal region (212, 431). p18 protein appears to play a role in facilitating the transfer of aminoacylated tRNA from the synthetase to the ribosome (446).

LysRS, along with other class IIb aaRSs, is characterized by three consensus motifs known as motifs 1, 2, and 3 (145). Figure 1.9A indicates the various domains in LysRS. Motif 1 contains a conserved Pro residue, and it is part of the dimer interface.

Motifs 2 and 3 together constitute the aminoacylation active site (64, 106, 263). Eukaryotic aaRSs, including LysRS, differ from their prokaryotic counterparts in having an N-terminal extension of these regions. For LysRS, there is a 65 amino acid N-terminal extension that function as a non-specific tRNA binding domain.

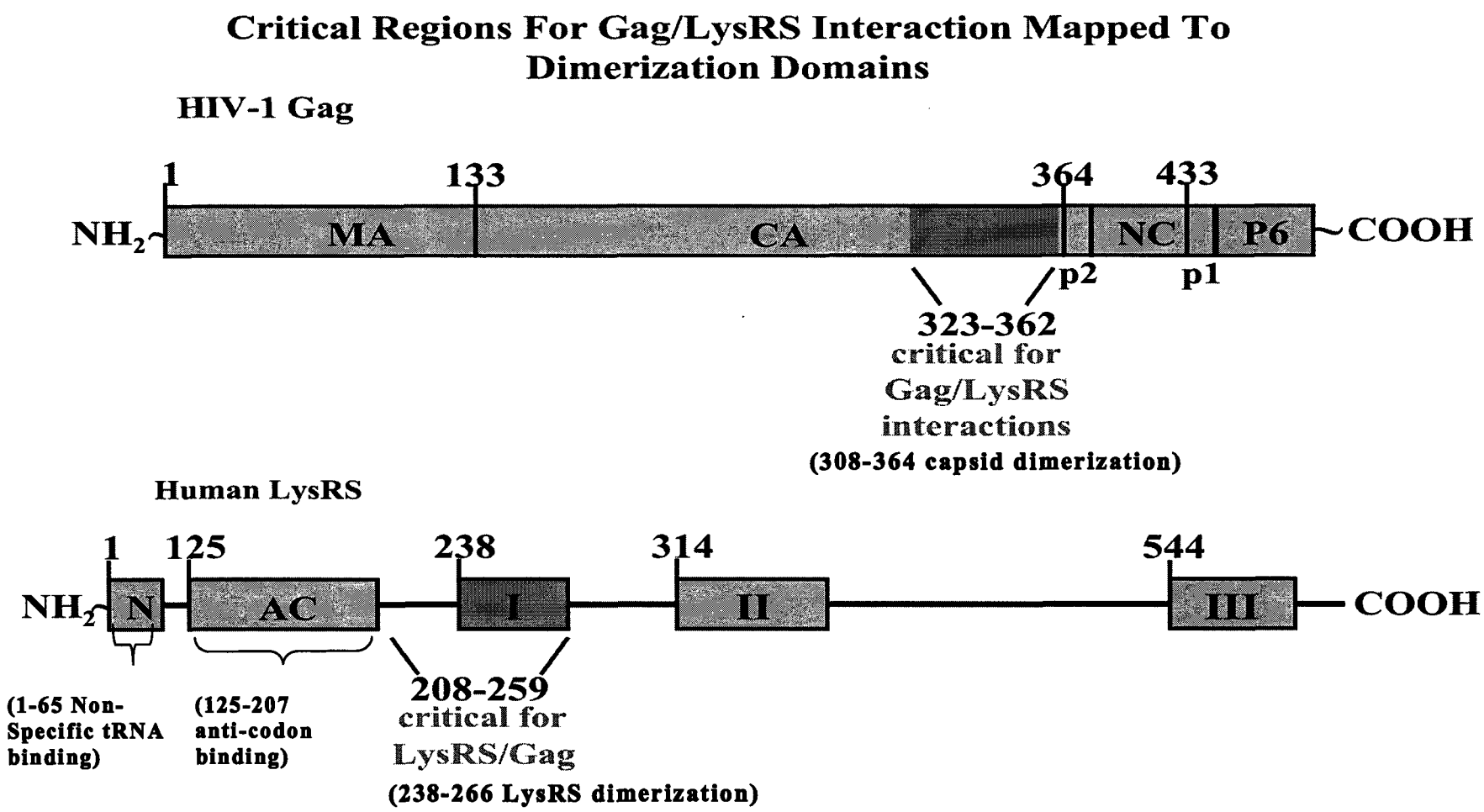
It has been shown that N-terminally truncated LysRS displays significantly weaker $tRNA^{Lys}$ binding affinity. Analysis using electrophoretic band shifting showed that N-terminally truncated hamster LysRS displayed a 100-fold lower affinity for $tRNA^{Lys}$, when compared to the native enzyme (161). Furthermore, LysRS also contains a $tRNA^{Lys}$ anticodon-binding domain that allows it to interact specifically with $tRNA^{Lys}$.

Figure 1.9. Interacting sites between human lysyl-tRNA-synthetase (LysRS) and HIV-1 Gag.

A. *In vivo* mapping of the interacting sites between Gag and LysRS (Adapted from (274)). Gag/LysRS interactions were determined via packaging of LysRS into Gag VLPs and the coimmunoprecipitation of Gag and LysRS, using mutant forms of both proteins. The determined sites included sequences for the dimerization sites in both LysRS (Motif I) and in capsid (C terminal domain (CTD)).

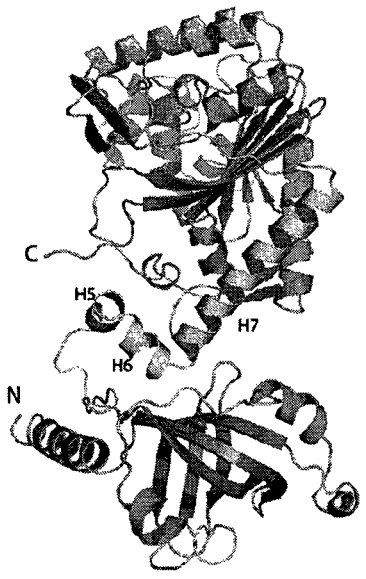
B. Ribbon structure of LysRS (Adapted from (412)) and the C-terminal domain of CA (Adapted from (185)), showing how they interact. *In vitro* mapping of interacting sites between purified HIV-1 Gag or capsid, and purified human LysRS, using fluorescence anisotropy and computational docking studies (Adapted from (308)). The results indicate that the interaction occurs between helix 7 in LysRS and helix 4 in the C-terminal domain of capsid in Gag. The putative CA (Gag) binding region in LysRS is in gold, and these helices are marked by an uppercase H. The CA-CTD helices are indicated by a lowercase h with the putative LysRS binding domain, h4, in green.

A

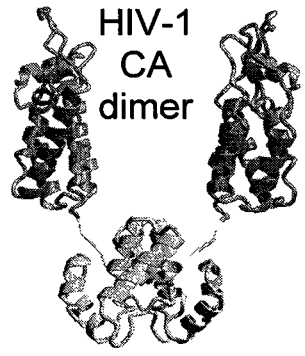

Critical Regions For Gag/LysRS Interaction Mapped To Dimerization Domains
HIV-1 Gag
1
133
364
433
NH2
MA
CA
NC
P6
COOH
p2
p1
323-362
critical for
Gag/LysRS
interactions
(308-364 capsid dimerization)
Human LysRS
1
125
238
314
544
NH2
N
AC
I
II
III
COOH
(1-65 Non-Specific tRNA binding)
(125-207 anti-codon binding)
208-259
critical for
LysRS/Gag
(238-266 LysRS dimerization)

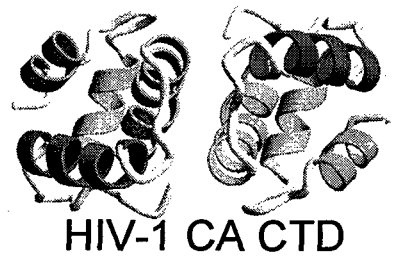
B

Homology model of human LysRS based on crystal structure of E. coli LysRS



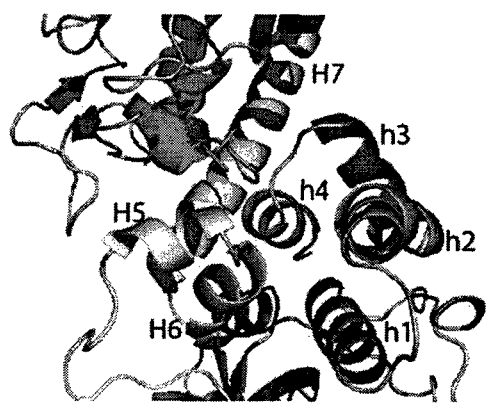
Onesti, S. et al. (1995)
Structure **3**: 163-176.





Gamble, T.R. et al. (1997)
Science **278**, 849–853.

Docking model of CA-CTD and LysRS depicting the interaction of helix 7 of human LysRS and helix 4 of HIV-1 CA



Kovaleski B. et al. (2007) *J Biol Chem* **282(44)**: 32274-9.

1.5.4.3 LysRS Incorporation into HIV-1

Human LysRS is incorporated into HIV-1 (70). From 50-100% (depending upon the cell type) of LysRS incorporated into HIV-1 appears to be smaller than cellular LysRS by approximately 5 KDa (70). The function of this truncation of LysRS is yet to be determined, but it could be essential either for facilitating $tRNA^{Lys3}$ annealing (by freeing the $tRNA^{Lys3}$ from the $tRNA^{Lys}$ packaging/annealing complex (since N-terminally truncated LysRS no longer binds $tRNA^{Lys3}$), or by optimizing the initiation of reverse transcription process (see Chapter 2). It might also facilitate deacylation of the $tRNA^{Lys3}$.

Viral LysRS does not appear to originate from any of its identified steady-state cellular compartments, which include the cytoplasmic HMW aaRS complex, nuclei, mitochondria, and cell membrane. Thus, removal of N- and C-terminal sequences which target LysRS to these compartments do not prevent the mutant LysRS from being incorporated into the virus (221). It has also been shown that the incorporation of LysRS into HIV-1 is very sensitive to the decrease in newly synthesized LysRS induced by small interfering RNA (siRNA) specific for LysRS. An 80% decrease in newly synthesized LysRS is accompanied by a similar decrease in viral LysRS at a time when the total cellular LysRS has only been reduced by only 20-25% (213).

Gag alone is capable of incorporating LysRS into Gag particles (70). *In vivo* and *in vitro* studies using wild type and mutant forms of LysRS have shown that Gag interacts directly with LysRS. The interaction of Gag with LysRS is very specific, i.e., when 12 aminoacyl tRNA synthetases (aaRSs) and the 3 non-synthetase proteins found in the HMW aaRS complex were tested for their presence in virions and for the ability to

be coimmunoprecipitated with Gag, only LysRS was found to be packaged into viruses or to interact with Gag (221). Domains critical for the Gag/LysRS interaction have been mapped *in vivo* to the C-terminal domain of CA sequences and motif 1 of LysRS, i.e., to regions including the dimerization domains of both CA and LysRS, respectively (274). Figure 1.9 A depicts the crucial regions for Gag and LysRS interactions. Both regions are rich in α–helices and sequences flanking these amino acids regions can be deleted from either molecule without affecting the Gag/LysRS interaction, supporting the observation that LysRS is packaged into Gag VLPs independently of its ability to bind $tRNA^{Lys}$.

Recent work using fluorescent anisotropy to study the *in vitro* interaction of LysRS with either Gag, CA, or the C-terminal domain (CTD) of CA, has more finely mapped the interacting sites to helix 7 of LysRS and helix 4 of the C-terminal domain of CA (307, 308)(Figure 1.9 B).

1.5.4.4 Formation of the $tRNA^{Lys}$ Packaging/Annealing Complex

GagPol is required for the selective packaging of $tRNA^{Lys3}$. VLPs composed only of Gag, however, will incorporate LysRS, demonstrating that the LysRS incorporation in HIV-1 occurs independently of $tRNA^{Lys3}$ packaging (358). This conclusion is further supported by the fact that N-terminally truncated LysRS that is unable to bind to $tRNA^{Lys3}$ is still packaged into the virus (274) . We propose that the selective packaging of $tRNA^{Lys}$ isoacceptors into virions results from Gag specifically binding to LysRS, but that GagPol is required to stabilize $tRNA^{Lys}$ in the packaging complex. Roughly equal numbers of LysRS and $tRNA^{Lys}$ molecules are incorporated into the virus (20-25

molecules of each (67, 257)).

Figure 1.10 presents a model for the formation of the $tRNA^{Lys}$ packaging/annealing complex. We propose that a Gag/GagPol/viral RNA complex interacts with a $tRNA^{Lys3}$/LysRS complex, with Gag interacting specifically with both LysRS and GagPol, and GagPol interacting with the $tRNA^{Lys3}$, and stabilizing its presence in the complex. In this model, we show monomeric LysRS interacting with unacylated $tRNA^{Lys3}$, but in fact the polymeric state of LysRS and the aminoacylation state of $tRNA^{Lys3}$ is not known. What is known is that while all detectable $tRNA^{Lys3}$ in the cytoplasm is acylated with lysine, all $tRNA^{Lys3}$ in the virus is not acylated (273). Only the dimer form of LysRS acylates $tRNA^{Lys}$, so we don't know if tRNA has not been acylated when it is packaged, or if requires a deacylation step. It has also been shown that expression of a mutant LysRS able to bind $tRNA^{Lys3}$, but not aminoacylate it, will still result in more $tRNA^{Lys3}$ being incorporated into the virus, indicating that aminoacylation of $tRNA^{Lys3}$ is not required for its packaging (68).

We discuss in detail the nature of the $tRNA^{Lys}$ packaging/annealing complex in the Discussion section in Chapter 2, and shall only briefly summarize it here. We have made an estimate of the polymerization number of Gag and GagPol in the $tRNA^{Lys}$ packaging/annealing complex, i.e., we estimate that a core Gag/GagPol/viral RNA complex is first formed through the interaction of 50 units of Gag, each unit being composed of a hexagon of Gag and a dimer of GagPol, resulting in 300 molecules of Gag and 100 molecules of GagPol, and that half of these hexagon units (25) contain one molecule each of $tRNA^{Lys}$ and one molecule of LysRS. Later addition of more Gag hexagons (without GagPol) to this core will give rise to the estimated 4000 molecules of

Gag found in the immature virion. This model predicts what we have found experimentally, i.e., that overexpression of LysRS in the cell can result in up to a doubling of viral incorporation of both LysRS and $tRNA^{Lys}$, without any change in the incorporation of GagPol (174).

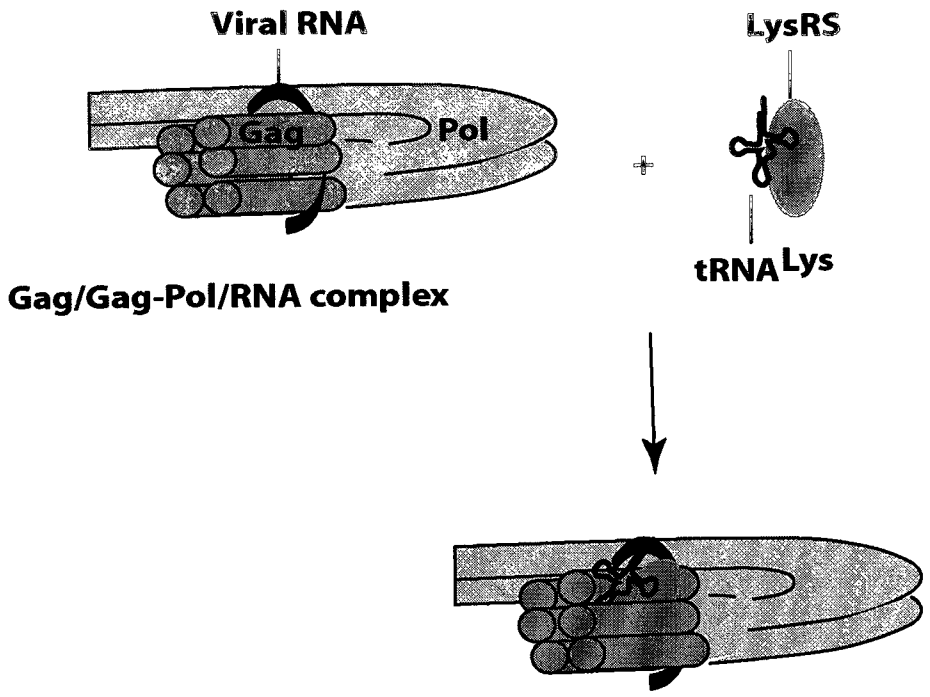
The model also predicts that GagPol has a conformation that results in its folding back so that Pol can interact with Gag. This too is discussed in detail in the discussion section in Chapter 2, and is based on work showing that Pol sequences alone can interact with Gag *in vivo*, and in fact, can replace the function of GagPol in selectively packaging tRNA (72). The advantages of such a conformation for the packaging and annealing of $tRNA^{Lys3}$ are also discussed in that section (see Figure 2.5 in Chapter 2).

The mechanism by which primer $tRNA^{Lys3}$ is selectively packaged into HIV-1 may also be used for avian retroviruses. For example, RSV uses $tRNA^{Trp}$ as the primer for reverse transcription, and we have found that TrpRS (and not LysRS) is selectively packaged into this virus (67). Interestingly, Mo-MuLV uses $tRNA^{Pro}$ as a primer, but shows both a reduced selective packaging of this tRNA, and contains neither ProRS, LysRS, nor TrpRS (331).

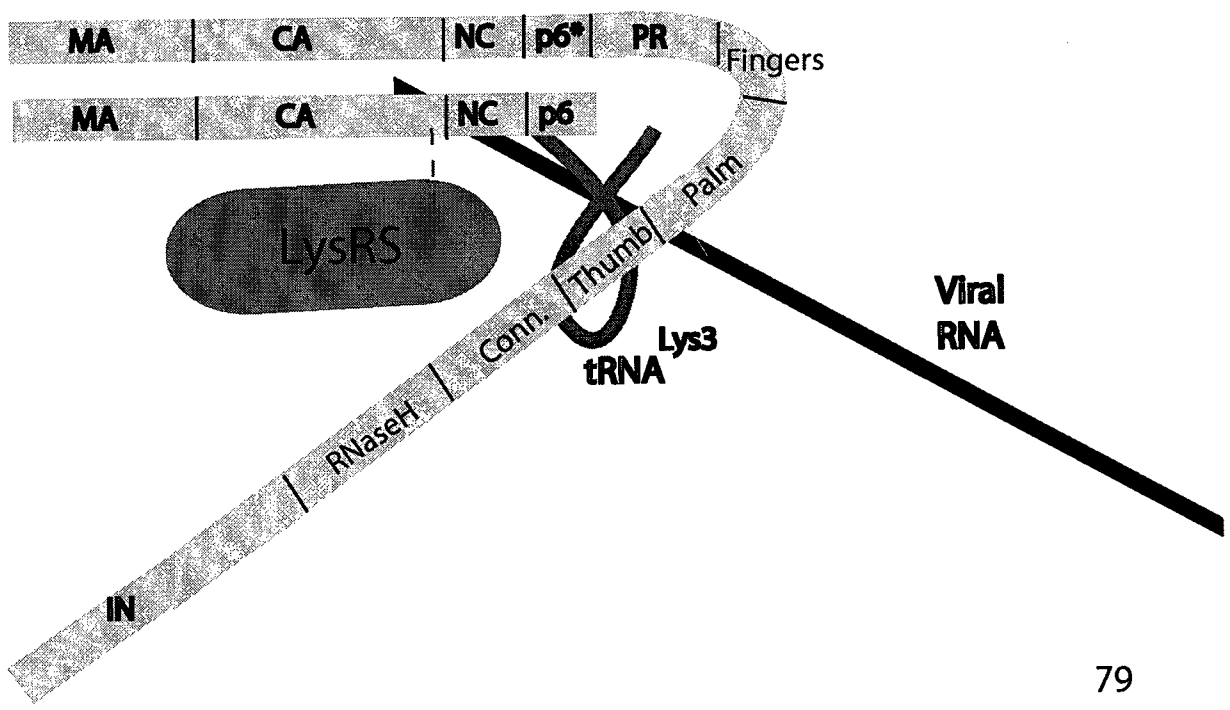
Figure 1.10 A Proposed model for the formation of the $tRNA^{Lys}$ packaging/annealing complex (Adapted from chapter 2, (479)).

(A) Formation of the $tRNA^{Lys}$ packaging/annealing complex. The picture shows a previously formed Gag/GagPol/viral RNA complex interacting with a $tRNA^{Lys3}$/LysRS complex, with Gag interacting with both GagPol and LysRS, and GagPol also interacting with $tRNA^{Lys3}$. The facts supporting this picture are described in detail in the text. **(B)** Proposed structure/function relationships existing between components of the $tRNA^{Lys}$ packaging/annealing complex. Although the actual conformation (s) of GagPol is not known, data in this paper and elsewhere suggests an interaction between Pol and Gag sequences. As described in chapter 2, the nature of this interaction is not clear, and while this work indicates the occurrence of a Gag/LysRS/Pol interaction, it appears that other interactions between Pol and Gag also occur. As described in chapter 2, we propose that these relationships will facilitate $tRNA^{Lys3}$ packaging and annealing, as well as facilitate retention of partially processed Pol during early stages of budding.

A


Viral RNA
LysRS
Gag
Pol
Gag/Gag-Pol/RNA complex
tRNA Lys
tRNA Lys
Packaging Complex

B


MA
CA
NC
p6*
PR
Fingers
MA
CA
NC
p6
LysRS
Palm
Thumb
Conn.
RNaseH
IN
Lys3
tRNA
Viral
RNA

1.6 Annealing of Primer $tRNA^{lys3}$ to HIV-1 Genomic RNA

1.6.1 Interactions between $tRNA^{Lys3}$ Primer and Genomic RNA

Packaged tRNA primer exists both in a free state and bound to the viral genome in retroviruses (439, 549). Although incorporation of primer tRNA into viruses occurs independently of genomic RNA packaging, it is not known when the tRNA primer is annealed to the PBS on the viral genome, i.e., before or after packaging (332, 338, 358). This event might occur when all the essential viral and cellular components are concentrated at the cell membrane, even before proteolysis is activated, since proteolysis of viral proteins is not required for $tRNA^{Lys3}$ annealing to the viral RNA genome (69). The 3′ terminal 18 nucleotides of the primer $tRNA^{Lys3}$ are complementary to the viral RNA 18 nucleotides composing the PBS that is located in the highly structured untranslated leader region at the 5′ end of the HIV-1 RNA genome (Figure 1.4). Several RNA structural models have been proposed for the PBS region (20, 26, 111, 465). Studies in HIV-1 (545) and MuLV-based retroviral vectors (352) have revealed that this complementarity must be maintained to accomplish efficient reverse transcription. Studies with both HIV-1 and avian leukosis virus (ALV) have revealed that PBS sequence is not in itself adequate to determine usage of a particular tRNA as the primer. Transfection of proviral DNAs with PBS sequences that are complementary to tRNAs other than the natural primer tRNA, yielded virus with initially slow replication kinetics in studies using either HIV-1 (117, 337, 546) or ALV (558). Nevertheless, the mutant genomes eventually revert back to their respective wild type PBS sequences, and the revertant virus then grow at wild-type rates. The reversion of altered PBSs in HIV and ALV to the natural wild type PBS suggests that there are factors other than just the PBS,

which influence the choice of the primer tRNA used, and in fact, RNA regions other than the PBS have been proposed to be involved in tRNA annealing (Figure 1.11).

The 5′ untranslated region (UTR) of the HIV-1 RNA genome includes the R, U5, and leader regions of the viral genome, terminating at the Gag initiation site (Figure 1.4). The 5′ UTR has been extensively studied, and a variety of structural models have been proposed (20, 26, 232) which consist of a series of stem/loop structures whose functions are connected with the recognition of various factors involved in different steps in the viral life cycle. These stem loops have been associated with the initiation (TAR) and termination and stability (poly A) of genomic RNA transcription, primer tRNA annealing (PBS region), genomic RNA dimerization (DIS) and packaging (Ψ), and the splice donor site in RNA splicing (SD). It has been suggested that these stem/loop structures are important in the primer tRNA/viral RNA interaction. For example, various predictions of the structure of the stem/loop containing the PBS have been proposed when it is complexed with the $tRNA^{Lys3}$ primer, two of which are shown in Figure 1.11 (A), proposed by Ehresmann's (264), and in Figure 1.11 (B) proposed by Berkhout's group (23).

The stem loop models have in common that they are composed of stems composed of U5 and leader sequences, with an intervening loop containing some (A) or all (B) of the PBS in a single stranded configuration. Several regions of $tRNA^{Lys3}$ are predicted to interact with several regions on the U5/PBS/leader structure, although other than the 3′ tRNA terminus interacting with the PBS, the hierarchal importance of the other interactions is controversial. In addition to the interaction of the 3′ region of $tRNA^{Lys3}$ with the PBS, model (A) shows other regions of tRNA, which may interact,

with viral RNA regions upstream of the PBS. These include the tRNA variable loop, the 3′ end of the anticodon stem, and the anticodon loop. The binding of continuous regions in $tRNA^{Lys3}$ to discontinuous regions in the viral RNA is accomplished by postulating stem/loop structures in the viral RNA, which bring the discontinuous binding regions in viral RNA closer to each other.

The model in Figure 1.11 A is currently in dispute, since the group that initially postulated these interactions later published a paper in which they found that while these interactions did occur in the HIV-1 Mal strain, some did not occur in the most common HIV-1 strains studied, such as HXB10 and NL 4-3 because some sequences in the Mal strain are absent in these other strains (199). For example, the interaction of the 4 consecutive U's in the $tRNA^{Lys3}$ anticodon loop with the 4 consecutive A's in the A rich loop at the top of the hairpin on the viral RNA (Figure 1.11 A), which was believed to be important for initiation of reverse transcription in HIV-1 Mal, does not occur in HXB10 or NL 4-3. It has also been reported that altering the PBS and the A-rich loop to be complementary to the 3′ terminal 18 nucleotides and anticodon, respectively, of some tRNAs, such as $tRNA^{His}$ (544), and $tRNA^{Met}$ (287), allows them to be used as primers in HIV-1. However, our laboratory reported that HIV-1 mutated this way in viral RNA uses these tRNAs very inefficiently as primers for reverse transcription, and replicate very poorly, probably because these tRNAs are not selectively packaged into the virus (552).

The model we currently favor for, the interaction between $tRNA^{Lys3}$ and viral RNA, is presented in Figure 1.11 B, which shows a different secondary structure for the U5/PBS/leader stem/loop. The model in Figure 1.11 B emphasizes, in addition to the 3′ terminus of $tRNA^{Lys3}$ interacting with the PBS, the importance of the interaction of the

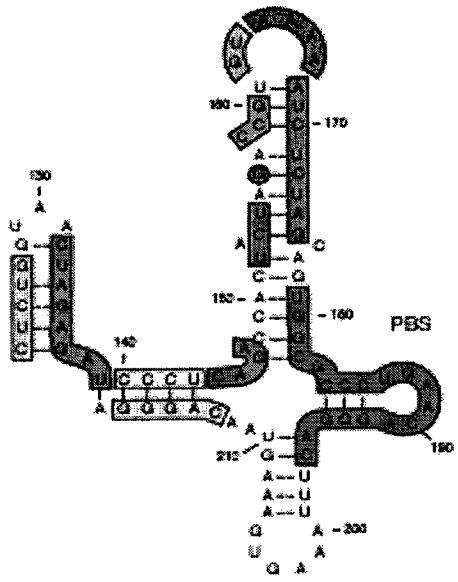
5′ portion of the TΨC arm in $tRNA^{Lys3}$ (also called the anti-PAS sequence) with viral RNA sequences (8 nucleotide motif) upstream of the PBS in the U5 region (+123 to +130) referred to as the primer activation site (PAS)(21).

Evidence from *in vitro* annealing of $tRNA^{Lys3}$ to mutant viral RNA indicates that while annealing of $tRNA^{Lys3}$ to viral RNA may only require annealing to the PBS, efficiency of the initiation of reverse transcription is optimal when the PAS double stranded region is denatured through mutation of the right strand, thereby allowing the anti-PAS sequence in the TΨC arm to anneal to the left strand of the PAS site (23). Mutations in the left strand strongly inhibit reverse transcription. The effects of these mutations upon reverse transcription was also confirmed *in vivo* by analyzing reverse transcription products from the mutant viral particles (22). Interaction of the 5′ portion of the TΨC arm with viral RNA has also been proposed for avian retroviruses, although the region in the avian viral RNA is postulated to be single stranded (5, 6).

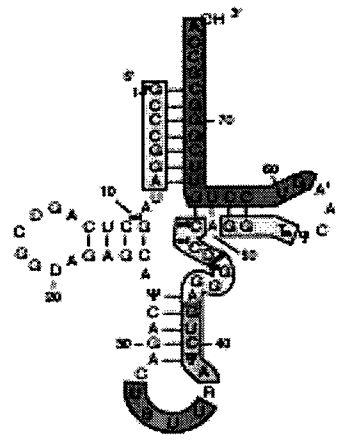
Figure 1.11. Proposed interactions between the $tRNA^{Lys3}$ primer and HIV-1 genomic RNA. (Adapted from (302)).

Two models for the interaction between $tRNA^{Lys3}$ and viral RNA. The 5′ region of viral genomic RNA containing the U5/PBS/leader sequence is shown in two different stem/loop configurations. Proposed hybridizing sequences between the viral genomic RNA and $tRNA^{Lys3}$ primer are identically colored. (A) The model proposed by Ehresmann's group (264). (B) The model proposed by Berkhout's group (23).

A

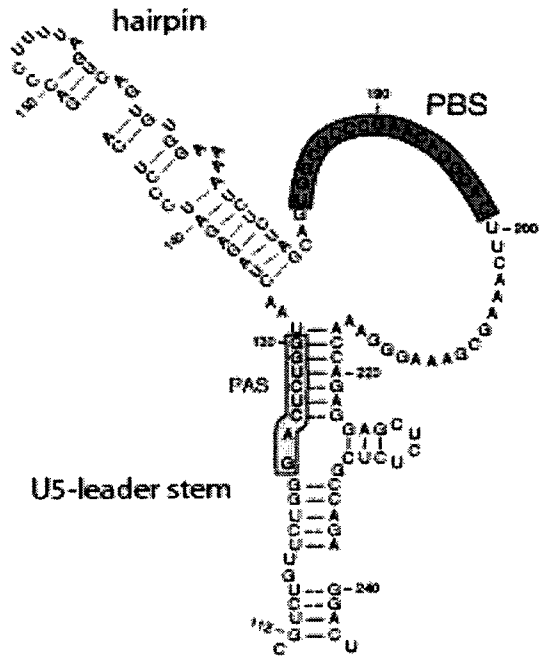


HIV-1 RNA

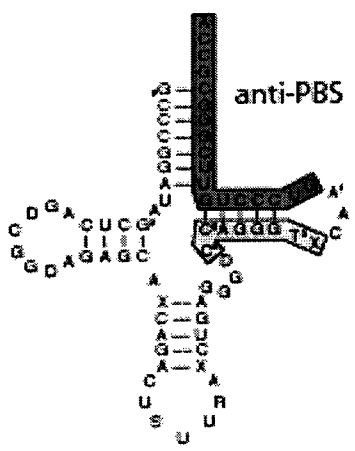


tRNA^{Lys3}

B



HIV-1 RNA



tRNA^{Lys3}

1.6.2 Viral Proteins that Facilitate tRNA^{lys3} Priming

1.6.2.1 Nucleocapsid (NC) and Gag

In vitro studies in HIV-1 have shown that mature NC facilitates the tRNA^{Lys3} annealing to *in vitro* transcribed genomic RNA sequences (17), possibly by unwinding the secondary structure of the stem/loop structures in the PBS region of the genomic RNA (266). It has been demonstrated by several groups that NC does not unwind the secondary structure of tRNA *in vitro*, and that the protein only has very subtle tertiary structural and helix destabilization effects on tRNA alone (73, 74, 228, 294, 533). Similar observations have been made in RSV and MoMuLV, where NC promotes the annealing of tRNA^{Trp} and tRNA^{Pro} to the PBSs of the RSV and MoMuLV genomic RNA, respectively (444).

It has been reported for both MoMuLV (119) and HIV-1 (120, 318) that the *in vitro* annealing of primer tRNA does not depend upon the presence of the zinc fingers, but depends upon the presence of the basic amino acids flanking the first, or only, zinc finger. Our laboratory has confirmed these observations *in vivo* for HIV-1 (66). In that report, it was also shown that mutations in the basic amino acids flanking the first zinc finger in Gag NC sequences had a much greater effect on inhibiting tRNA^{Lys3} annealing than similar mutations in the NC sequence in GagPol, indicating an important role for the NC domain in Gag for promoting tRNA^{Lys3} annealing. In fact, Gag alone can facilitate tRNA^{Lys3} annealing to viral RNA *in vitro* (154) , and neither its processing, nor that of GagPol, is required for annealing to the PBS *in vivo* (69). Nevertheless, although the annealing of tRNA^{Lys3} to the PBS *in vivo* does not require precursor proteolysis,

exposure of Gag-annealed $tRNA^{Lys3}$ to mature NC is required for optimum placement, i. e., for the most efficient initiation of reverse transcription (69). Thus, when total viral RNA isolated from either wild type or protease-negative (Pr-) HIV-1 was used as the source of primer $tRNA^{Lys3}$/viral RNA template in an *in vitro* RT reaction, the incorporation of the first nucleotide, dCTP, was 3 times more efficient for wild-type than Protease negative (Pr-) HIV-1 at low dCTP concentrations. Transient exposure to NC of the $tRNA^{Lys3}$/viral RNA complex isolated from Pr- HIV-1 increased efficiency of dCTP incorporation to wild-type levels, while transient exposure to Gag had no effect. Thus, although mature NC does not appear to be required for the initial annealing of $tRNA^{Lys3}$, it does appear to be required to optimize the final placement of the primer $tRNA^{Lys3}$ onto the PBS. In Chapter 3, we have further examined the roles of Gag and NC in $tRNA^{Lys3}$ annealing, and we demonstrate the weaker annealing of Gag-annealed $tRNA^{Lys3}$ by another assay that measures the PBS occupancy by $tRNA^{Lys3}$. We have previously reported that APOBEC3G (A3G) can inhibit $tRNA^{Lys3}$ annealing both *in vivo* and *in vitro* (215), and in Chapter 3, we have used A3G to further probe the relationship between Gag and NC $tRNA^{Lys3}$ annealing, based upon our observations that A3G does not inhibit annealing facilitated by Gag, but does inhibit NC-facilitated annealing.

1.6.2.2 Reverse Transcriptase (RT)

RT or RT sequences within GagPol plays a role in facilitating tRNA annealing to viral genomic RNA in two ways. First, because RT sequences are required for the selective packaging of primer, and Gabor *et.al* (174) have shown a direct correlation between the amount of $tRNA^{Lys3}$ packaged into the virus and the amount of $tRNA^{Lys3}$

annealing that occurs. The reduction in annealing observed in RT-negative ASV (439) and HIV-1 (358) is likely to be due to the observed reduction in packaging of $tRNA^{Trp}$ and $tRNA^{Lys3}$, respectively. However, RT sequences appear to also be directly involved in regulating annealing independently of primer tRNA. The RT thumb domain is required for the interaction of GagPol with $tRNA^{Lys}$, and its incorporation into virions (296). It has been shown that virions containing GagPol with C terminal deletions up through the RT connection domain, while still selectively packaging $tRNA^{Lys}$, show a 75% reduction in $tRNA^{Lys3}$ annealing, thus indicating a role of RT in annealing independent of $tRNA^{Lys3}$ incorporation (71).

1.6.3 Does LysRS Directly Facilitate $tRNA^{Lys3}$ Priming?

LysRS plays an important role in the selective packaging of $tRNA^{Lys3}$ into HIV-1 through its specific interaction with both $tRNA^{Lys3}$ and the C-terminal domain of CA sequences in Gag (274). The amount of $tRNA^{Lys3}$ annealing is directly proportional to the amount of $tRNA^{Lys3}$ packaged into the virion, with increased $tRNA^{Lys3}$ packaging and annealing accompanying overexpression of LysRS (174), and decreased $tRNA^{Lys3}$ packaging and annealing resulting from decreased LysRS incorporation due to exposure of cells to siRNA LysRS (213). However, LysRS may also play a more direct role in the annealing of $tRNA^{Lys3}$. While the thumb domain in RT is required for $tRNA^{Lys3}$ packaging, the connection domain is required for $tRNA^{Lys3}$ annealing. Thus, viruses in which GagPol has a C-terminal deletion up through the connection domain will still selectively package $tRNA^{Lys3}$, but will not anneal it to the viral RNA (296). The role of the connection domain in $tRNA^{Lys3}$ annealing is not clear, but the work described in

Chapter 2 shows that LysRS binds to Pol through sequences in the CD/RN region, suggesting the possibility that this interaction may help facilitate annealing.

1.6.4 Inhibition of $tRNA^{Lys3}$ Annealing by APOBEC3G

1.6.4.1 APOBEC3G Structure and Properties

Human APOBEC3G, or A3G (Human Apolipoprotein B mRNA-Editing Enzyme-Catalytic Polypeptide-Like-3G) is an endogenous cellular inhibitor of HIV-1 infectivity (231, 367, 585), and is a member of an RNA/DNA cytidine deaminase superfamily, the APOBEC3 (hA3) family. Human chromosome 22 contains a gene cluster of 7 members of the APOBEC3 (hA3) family that include A3A, A3B, A3C, A3DE, A3F, A3G, and A3H (272). All members of the APOBEC3 family have either one (A3A, A3C, A3H) or two (A3B, A3DE, A3F, A3G) zinc-binding motifs or cytidine deaminase domains (CDs) with the conserved sequence His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys (272, 551). Five of the hA3 proteins (A3B, A3C, A3DE, A3F, and A3G) have been shown to deaminate cytidine in DNA and to have anti-HIV-1 activity (105, 112). A3F and A3G are the most potent anti-HIV-1 compounds (32, 112, 128, 317, 471). A3A is inactive as an anti-HIV agent, although it appears to be a powerful inhibitor of retrotransposition by the intracisternal A-particle retrotransposon in human cells, using an unknown non-editing mechanism (35).

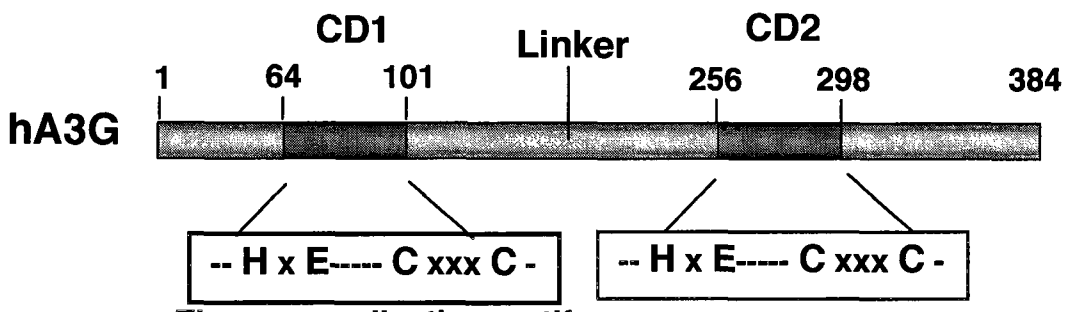
A3F and A3G are expressed in the major target cells of HIV-1, T4 lymphocytes and macrophages (32, 128, 343, 560). A3F (373 amino acids) and A3G (384 amino acids) are similar in length, with a 49% amino acid identity, and show only one amino

acid difference in the first N-terminal 60 amino acids (590). A3G and A3F edit (i.e., deaminate dC to dU) within the viral DNA sequence context CC* and TC*, respectively, where C* is the base targeted for deamination (32, 128, 231, 343, 560).

A3G has two zinc-binding domains, connected by a linker region (Figure 1.12) (272, 551). These two zinc-binding motifs or CDs perform quite distinct functions despite their homology; the N-terminal motif (CD1) promotes RNA binding and virion encapsidation (268, 399, 402), while the C-terminal motif (CD2) confers deoxycytidine deaminase activity (220, 268, 399, 402) and sequence specificity for modifying the single strand DNA substrate (32, 231, 343, 367, 560, 585).

Figure 1.12 Organization of APOBEC3G.

A3G contains two zinc-binding motifs or CDs with the conserved sequence His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4} –Cys, separated by a linker region. They are also referred to as catalytic domain 1 (CD 1) and 2 (CD2), although it is not clear if CD1 has any deaminase function. Thus, these two are associated with quite distinct functions. The N-terminal motif (CD1) promotes RNA binding and APOBEC3G encapsidation, while the C-terminal motif (CD2) confers deoxycytidine deaminase activity and sequence specificity for modifying the single strand DNA substrate.



Zinc++ coordination motif

1.6.5.1.2 APOBEC3G and Viral Infectivity Factor (Vif)

HIV-1 combats the antiviral effect of A3G through the expression of the viral Vif protein, a 190-240 amino acids, 23 KDa, accessory protein that is encoded by all of the lentiviruses except for equine infectious anemia virus (EIAV)(125, 159, 175, 195, 286, 326, 357, 502, 503, 507, 522, 541). Vif is required for HIV-1 productive infection in non-permissive cells (176, 201, 357) such as primary CD4+ T-lymphocytes, H9, macrophages and dendritic cells expressing A3G (79-81, 312). However, it is not required in permissive cells such as SupT1 and Jurkat cells (159, 175, 522). Replication of HIV-1 in primary cells is dependent on the expression of Vif proteins, which binds to and counteracts the activity of the hosts A3G protein (32, 497).

Vif prevents the incorporation of A3F and A3G into HIV-1 by binding to these molecules (32, 317, 560, 590). It then targets them for proteasomal degradation by also binding to a Cul5E3 ligase, a component of the Elongin B/C complex of the ubiquitination machinery, thereby inducing the ubiquination of A3G and A3F, and their subsequent degradation by the proteosome (98, 304, 369, 370, 385, 386, 520, 559, 579). Therefore, in the absence of Vif, A3G binds to Gag to become incorporated into viral particles (65, 491, 524, 584).

Mutational analyses have shown that the N-terminal region of Vif binds to the N-terminal region of A3G (amino acids 54-124)(98, 370, 504, 559). However, it is the N-terminal region (amino acids 105-245) of the linker sequence between the two CDs that is required for Vif mediated degradation. Furthermore, the C-terminal region of the linker sequence, which does not bind to Vif, is also involved in Vif mediated

degradation (586).

1.6.5.1.3 APOBEC3G Incorporation into HIV-1

The encapsidation of A3G requires its interaction with the NC region of HIV-1 Gag (8, 65, 354, 491, 584). This interaction is RNA-dependent (16, 53, 292, 293, 491, 510, 524, 548, 584), but whether this reflects an RNA molecule serving as a bridge between the two molecules, or rather a direct interaction between Gag and A3G in which the required conformation of either molecule for this interaction is RNA-dependent, is unknown (105, 581). The Gag binding site in A3G have been mapped to the N-terminal part (residues 104-156) of the linker sequence between the two CDs (65)(Figure 1.12).

Two cellular forms of A3G-containing complexes have been identified in the cell: a high molecular mass (HMM) and a low molecular mass (LMM) complex. The HMM complexes are enzymatically inactive and can be artificially converted into the enzymatically active LMW complexes by treatment with RNase H suggesting that RNA plays a pivotal role in HMM A3G complex assembly (81). A direct correlation between A3G viral incorporation and HMW complex formation has been reported, suggesting that HMW complex might act as the cellular source of viral A3G (65).

1.6.5.1.4 APOBEC3G Functions

A3G potently interferes with virus replication (289, 347, 369, 370, 498, 520, 579). Vif-negative viruses produced in cells expressing A3G or A3F incorporate increased amounts of A3G or A3F compared to Vif-positive viruses, which results in a strong inhibition of new viral DNA synthesis (> 95% reduction) upon new infection by

these viruses. How does A3G affect viral DNA production? Because the small amount of minus strand cDNA that is made in newly-infected cells contains 1-2% of the cytosines mutated to uracils (231, 322, 367, 585), it has been suggested that A3G anti-HIV-1 activity stems from its ability to form dU by deaminating dC in the minus strand cDNA, thereby facilitating the DNA's degradation by the DNA repair system.

However, accumulated recent evidences indicate that a deaminase-independent mechanism might also be involved in A3G's antiviral activity because of the following facts: first deamination activity is not absolutely linked with antiviral activity against HIV-1 (31, 247, 268, 355, 399, 402, 419, 501). For example, the cellular uracil DNA glycosylase, UNG2, is incorporated into HIV-1 by binding to both Vpr and integrase (496, 563). UNG2 is a major cellular enzyme that removes uracil from DNA, thereby leaving an abasic residue in the DNA phosphodiester backbone, which can be excised by an endnuclease. If no complementary DNA strand is present, this could lead to DNA degradation. However, a reduction in UNG2 incorporation into HIV-1 containing A3G, using either an UNG2 inhibitor, UGI, or virus-producing cells lacking endogenous UNG2 activity, had no effect on the antiviral capacity of A3G, i.e., in the presence of A3G, viral infectivity and resulting synthesis of viral DNA are reduced equally with or without viral UNG2 (284).

Furthermore, several reports have shown that mutant A3G (81, 214, 402) and mutant A3F (246) that have lost their cytidine deaminase activity still show strong anti-HIV-1 activity, and A3G can also inhibit Hepatitis B virus (HBV) replication with little or no editing (404, 472, 535). Also other APOBEC proteins block replication of mouse mammary tumor virus (411) and several retrotransposons (35, 36, 77, 247, 396) in the

absence of editing activity.

Therefore, the reduced viral DNA production in the presence of A3G may actually be due to reduced reverse transcription (non-deaminase related function) rather than degradation of DNA transcripts (deaminase related function).

1.6.5.1.5 APOBEC3G Effects on Reverse Transcription and $tRNA^{Lys3}$ Annealing

Several mechanisms responsible for the reduced reverse transcription by A3G have been suggested by our laboratory (65, 214, 215) and others (379), and they involve a cytidine-deaminase-independent reduction both in minus and plus strand DNA transfer steps occurring during reverse transcription (339), and in $tRNA^{Lys3}$ annealing to viral RNA (215, 216). It was reported that $tRNA^{Lys3}$ priming of reverse transcription is inhibited in the naturally nonpermissive H9 and MT2 cell lines, compared with priming in the permissive MT4 cell line. Levels of inhibition of $tRNA^{Lys3}$ priming similar to that found in H9 cells can be observed in 293T cells expressing exogenous A3G (214). Furthermore, *in vitro* inhibition of $tRNA^{Lys3}$ priming by purified A3G was shown to be the result of reduced $tRNA^{Lys3}$ annealing, and it required an interaction with NC (215). The A3G/NC interaction depends upon the presence of RNA. The reaction measuring this interaction contained no specifically added RNA, and since the NC used does not contain RNA, this RNA must be associated with A3G. It is of interest that both $tRNA^{Lys3}$ annealing to viral RNA and DNA strand transfers are facilitated by the viral protein, NC (101, 115, 242, 330, 455). In Chapter 3, the discovery that A3G can only inhibit NC-facilitated $tRNA^{Lys3}$ annealing, and not Gag-facilitated $tRNA^{Lys3}$ annealing, even though both Gag and NC interact with A3G (215), is used to further examine the roles played by

Gag and NC in the annealing of $tRNA^{Lys3}$ to viral RNA, both *in vivo* and *in vitro*.

CHAPTER II

Interactions of reverse transcriptase sequences in Pol with Gag and LysRS in the HIV-1 $tRNA^{Lys}$ packaging/annealing complex

(This chapter was adapted from an article published in Virology (2008)(479))

2.1 Preface

Figure 1.10A shows our model of how we believe the $tRNA^{Lys}$ packaging/annealing complex is formed, while the structural relationships between the various components in the complex are presented in Figure 1.10B. We propose that within this complex $tRNA^{Lys3}$ needs to be transferred both from LysRS to the thumb domain of RT, and from RT to the PBS of the viral RNA. The model as drawn in Figure 1.10B suggests that there may be an interaction between LysRS and Pol that might facilitate either of these transfers. In Chapter 2, representing a article from a published in virology (479), we describe our investigations showing that such an interaction between LysRS and Pol does exist, and we show that the LysRS binding site on Pol is contained within the RNAseH/connection domain of RT. We also show that the Gag/LysRS/Pol bridge is not required for an observed interaction between Gag and Pol. Combining our mapping studies with data reported elsewhere that indicates the role of RT structural domains in both $tRNA^{Lys3}$ packaging and annealing, we conclude that the LysRS/Pol interaction is probably not involved in facilitating transfer of $tRNA^{Lys3}$ from LysRS to Pol, but it may play a role in the transfer of $tRNA^{Lys3}$ from RT to the PBS in the viral RNA.

2.2 Abstract

During HIV-1 assembly, $tRNA^{Lys3}$, the primer for reverse transcriptase (RT) in HIV-1, is selectively packaged into the virus due to a specific interaction between Gag and lysyl-tRNA synthetase (LysRS). However, while Gag alone will incorporate LysRS, $tRNA^{Lys3}$ packaging also requires the presence of RT thumb domain sequences in GagPol. The formation of a $tRNA^{Lys}$ packaging/annealing complex involves an interaction between Gag/GagPol/viral RNA and LysRS/$tRNA^{Lys}$, and herein, we have investigated whether the transfer of $tRNA^{Lys3}$ from LysRS to RT sequences in Pol by a currently unknown mechanism is facilitated by an interaction between LysRS and Pol. We demonstrate that, in addition to its interaction with Gag, LysRS also interacts with sequences within the connection/RNaseH domains in RT. However, cytoplasmic Gag/Pol interactions, detected by either coimmunoprecipitation or incorporation of Pol into Gag viral-like particles, were found to be insensitive to the overexpression or underexpression of LysRS, indicating that a Gag/LysRS/RT interaction is not essential for Gag/Pol interactions. Based on this and previous work, including the observation that the RT connection domain is not required for $tRNA^{Lys3}$ packaging, but is required for $tRNA^{Lys3}$ annealing, a model is proposed for a $tRNA^{Lys}$ packaging/annealing complex in which the interaction of Gag with Pol sequences during early viral assembly facilitates the retention in budding viruses of both $tRNA^{Lys3}$ and early Pol processing intermediates, with $tRNA^{Lys3}$ annealing to viral RNA further facilitated by the LysRS/RT interaction.

2.3 Introduction

In lentiviruses, including HIV-1, $tRNA^{Lys3}$ serves as the primer tRNA for the reverse transcriptase (RT)-catalyzed synthesis of minus strand strong stop DNA (328, 360). $tRNA^{Lys3}$ and the other major $tRNA^{Lys}$ isoacceptors, $tRNA^{Lys1}$ and $tRNA^{Lys2}$ (referred to collectively as $tRNA^{Lys1,2}$, since these two isoacceptors differ by only one base pair in the anticodon stem) are selectively packaged into the virus (358). While the function of $tRNA^{Lys1,2}$ in HIV-1 is not known, the selective packaging of primer $tRNA^{Lys3}$ is required for optimizing both the annealing of $tRNA^{Lys3}$ to viral RNA and the infectivity of the HIV-1 population (174). The major structural protein in HIV-1, Gag, is capable, with the help of genomic or cellular RNA, of forming extracellular Gag viral-like particles (VLPs) (58, 210, 295, 397), but these Gag VLPs do not selectively incorporate $tRNA^{Lys}$ unless GagPol is also present (358).

The interaction between GagPol and $tRNA^{Lys3}$ involves the thumb domain (TH) sequences in RT (296), and while GagPol plays the role of stabilizing the $tRNA^{Lys}$ in the Gag/GagPol/viral RNA complex, Gag plays the important role of selecting $tRNA^{Lys}$ isoacceptors for incorporation into HIV-1. It does so by specifically binding the $tRNA^{Lys}$ binding protein, lysyl-tRNA synthetase (LysRS), and this protein is incorporated into both HIV-1 and Gag VLPs (70). The interaction of Gag with LysRS is very specific, i.e., of 12 aminoacyl tRNA synthetases (aaRSs) and 3 related proteins tested, only LysRS is packaged (221). Domains critical for the Gag/LysRS interaction have been mapped to include the dimerization domains of both LysRS and capsid (CA) (274). Recent work using fluorescent anisotropy to study the *in vitro* interaction of LysRS with either Gag, CA, or the C-terminal domain of CA, has more finely mapped the interacting sites to

helix 7 of LysRS and helix 4 of the C-terminal domain of CA (307, 308). Viral LysRS does not appear to originate from any of its identified steady-state cellular compartments, which include a cytoplasmic high molecular weight aaRS complex, nuclei, mitochondria, and cell membrane. Instead, newly-synthesized LysRS appears to interact with Gag before entering any of these compartments (213, 221). The packaging of $tRNA^{Lys}$ isoacceptors requires interaction with LysRS (273), but not aminoacylation of the tRNA (68). Based upon these findings, we have postulated a model for the formation of a $tRNA^{Lys}$ packaging/annealing complex, in which a Gag/GagPol/viral RNA complex interacts with a $tRNA^{Lys}$/LysRS complex, with Gag interacting with both GagPol and LysRS, and GagPol also interacting with $tRNA^{Lys}$ through the TH domain in RT (see Figure 5A).

The *in vivo* interaction of Gag with GagPol has been well documented (430, 505, 506, 513), and it is believed that GagPol is carried into the assembling Gag particle by interaction of Gag sequences in GagPol with those in Gag. Based on the interactions occurring between Gag molecules, the interaction between Gag and Gag sequences in GagPol may only involve the C-terminal domain of CA and SP1 sequences. Thus, cryoelectron microscopy has indicated that in immature virions, Gag is radially distributed in the virion, with the N-terminal sequences within the matrix (MA) domain associated with membrane, and the C-terminal sequences coding for SP2-nucleocapsid (NC)-p6 nearest the center of the virion (173, 562). Images of immature virions have suggested that Gag molecules may be arranged in interacting hexagonal bundles, with a Gag molecule at each vertex of the hexagon (41, 378, 401, 571), which is supported by work studying the *in vitro* assembly of Gag (261). The hexagonal order, however,

appears to be primarily at the level of CA and SP1, and is not found at the levels N- or C-terminal to these regions (41, 378, 401, 571), suggesting that Gag/Gag interactions may occur primarily at the CA/SP1 region.

Interactions between individually expressed HIV-1 Pol and Gag molecules have also been reported, reminiscent of the intermolecular interaction between Pol and Gag in human foamy viruses, which are expressed from separate mRNAs (516). Expression of HIV-1 Pol and Gag from separate plasmids in 293T cells not only results in the incorporation of Pol into Gag VLPs, but Pol can replace GagPol in facilitating the selective incorporation of $tRNA^{Lys}$ into the Gag VLPs (72). A recent report also indicates that RT sequences alone can be incorporated into Gag VLPs, with apparent interactions occurring between matrix and p6 sequences in Gag and the TH domain in RT (342).

Because the formation of the $tRNA^{Lys}$ packaging/annealing complex appears to require a transfer of $tRNA^{Lys3}$ by an unknown mechanism from LysRS to RT sequences in Pol, we have investigated whether there is a direct interaction between LysRS and Pol, and in this work, we demonstrate the existence of such an interaction.

2.4 Experimental Procedure

2.4.1 Plasmid construction

The plasmids used in this study are listed in Table 2.1. GagFS- codes for all HIV-1 proteins except GagPol, and was constructed as previously described (341). It contains mutations at the GagPol frame shift site (2082-TTTTTT-2087 replaces 2082-CTTCCT-2087) that prevent frame shifting. RSV.Gag.P-, previously named pSV.Myr1.3h, was a gift from R. Craven and J. Wills (University of Pennsylvania, Philadelphia), and was constructed as previously described (99). It encodes a myristylated RSV Gag protein and the first seven amino acids of RSV protease. Except for Gag made from GagFS-, all HIV-1 proteins expressed in cells (Gag, GagΔP6, Pol, RT, and corresponding truncated fragments), were expressed from codon-optimized mRNAs. These proteins have identical amino acid sequences to their viral counterparts, but the mRNA coding for them have had their codons optimized for mammalian cell codon usage, which results in more efficient translation and protein production and also makes nuclear export of these mRNAs Rev-independent through modification of the multiple inhibitory sequences (256). The construction of Gag plasmid was previously described (256). The GagΔp6 plasmid was PCR-amplified from Gag plasmid and digested with SalI and XbaI, whose sites were introduced in each of the following primers:

5′- ATAATAGTCGACATGGGCGCCCGCGCCAGCGTG-3′(sense);

5′-GCGGCGTCTAGATTAAAAATTCCCTGGCCTTCC-3′ (anti-sense). This PCR fragment was cloned into the SalI and XbaI sites of pNGVL-3 plasmid backbone after the removal of hGag.

hPol cDNA was PCR-amplified from a plasmid coding for hPol (a gift from Y. Huang and G. Nabel, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland (256)), and digested with SalI and EcoRI, whose sites were introduced into each of the PCR primers that also contained V5 sequences, as previously described (72). Pol cDNA (coding for the first 1001 amino acids of Pol) was then cloned into a plasmid pNGVL-3 (a gift from Y. Huang and G. Nabel) from which GagPol had been removed, and Pol fragments were amplified using specific PCR primers. Both Pol and Pol fragments contain C-terminal V5 sequence, and an inactive protease due to an R42G mutation in the active site in protease. Pol fragments were constructed using Pol as a template and the coding regions are indicated for each fragment as follows: PR-C (amino acids 58-1001) a fragment that is missing P6*; FP-C fragment (amino acids 155-1001) missing P6* and protease; TH-C fragment (amino acids 238-1001) missing P6*, Protease, and finger/palm domains; CD-C (amino acids 476-1001) fragment missing P6*, protease, and the finger/palm and thumb domains; N-RN (amino acids 1-714), a fragment missing integrase. RT (amino acids 155-714) was amplified from the N-RN fragment. Other RT fragments were amplified from RT and the coding regions are indicated as follows: PA-CD (amino acids 155-584); FP-TH (amino acids 155-475); TH-CD (amino acids 397-584); CD-RN fragment (amino acids 476-714). All plasmids were verified by sequencing (McGill University and Genome Quebec Innovation Centre, Montreal, Quebec). Plasmid pLysRS contains cDNA encoding full-length (1 to 597 amino acids) human LysRS, as previously described (274), and expresses wild-type LysRS protein in transfected 293T cells.

2.4.2 Production of Gag viral-like particles (VLPs) and incorporation of Pol or RT.

HEK-293T cells were grown in complete Dulbeco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS), 100U of penicillin, and 100ug of Streptomycin per ml, were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cell culture supernatant was collected 48 h post-transfection. Gag viral-like particles (VLPs) were pelleted from culture medium by centrifugation in a Beckman SW41 rotor at 35,000 rpm for 1 h through a 20% sucrose cushion. The pellet of purified Gag VLPs was resuspended in lysis buffer (RIPA buffer: 10 mM Tris, pH 7.4; 100 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 1% NP-40; 2 mg of aprotinin/ml; 2 mg of leupeptin/ml; 1 mg of pepstatin A/ml; 100 mg of phenylmethylsulfonyl fluoride/ml).

2.4.3 Coimmunoprecipitation of LysRS or Gag with Pol or RT.

Transfected 293T cells, removed 48 hrs. post-transfection, were washed with PBS, and lysed in 350 μl RIPA buffer. Insoluble material was pelleted at 10,000 rpm for 30 minutes in a IEC MICROCL 21 microcentrifuge, and the supernatant was used for coimmunoprecipitation studies. 20 μl anti-LysRS and 400 μl of 50% (w/v) protein A-Sepharose (Pharmacia) were incubated together in 10 ml of 0.2 M triethanolamine pH 9. Dimethyl pimelimidate (DMP) cross-linker (Pierce) was then added to a final concentration of 20 mM, and the mixture was incubated overnight at 4^{o}C. The beads were then washed three times with 10 ml of 0.2 M triethanolamine pH 9. Equal amounts of protein in cell supernatant (approximately 200 - 500 μg, as determined by the BioRad

assay) were incubated with 40 µl antibody cross-linked to protein A-Sepharose overnight at 4°C. The immunoprecipitate was then washed three times with Net gelatin buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCL, 0.1% NP-40, 1mM EDTA, 0.25% gelatin). After the final supernatant was removed, 50 µl of 2X sample buffer (120 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, and 0.02% bromphenol blue) was added and the precipitate was then boiled for 10 minutes to release the precipitated proteins. After microcentrifugation, the resulting supernatant was analyzed using Western blots.

2.4.4 RNAse A and T1.

Cell lysates were immunoprecipitated as described above and then the immunoprecipitates were treated with RNAse A (Fermentas) and T1 (Ambion) for 2 hrs at 37 °C, washed three times with Net gelatin buffer, then resuspended in 2X loading dye and followed by western blots as described below.

2.4.5 Protein analysis.

Viral and cellular proteins were extracted with RIPA buffer, resolved by SDS-PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amersham Pharmacia Biotech). Detection of protein by Western blotting utilized monoclonal antibodies that are specifically reactive with HIV-1 RT p66 (HIV-1 RT monoclonal antibody (7E5), Cat. #7372, NIH AIDS Research and Reference Reagent Program), a polyclonal antibody for human LysRS (Pocono Rabbit Farm and Laboratory, Inc.), and monoclonal antibodies for V5 (Sigma), ß-actin (Sigma), GAPDH (Santa Cruz), HIV-1 capsid (Zepto Metrix), and RSV CA (a gift from Dr. Volker M. Vogt, Cornell University). Detection of proteins was performed by enhanced

chemiluminescence (NEN Life Sciences Products) using the following secondary antibodies obtained from Amersham Pharmacia Biotech: anti-mouse (for HIV-1 CA, RT, V5, ß-actin, and GAPDH) and anti-rabbit (for LysRS, and RSV CA).

2.4.6 siRNA transfection.

T3 siRNA to LysRS and control siRNA to luciferse were constructed as previously described (213). At 24 h before transfection, HEK-293T cells were trypsinized and plated in six-well plates at 1.0 X 10^5 cells per well in 2 ml of DMEM supplemented with 10% FBS. The cells were incubated at 37^0C in a CO_2 incubator until 60-80% confluent. These HEK-293T cells were transfected with siRNA, using cationic lipid complexes that were prepared by incubating 50 pmol of indicated siRNA with 5 µl of DMRIE-C reagent (Invitrogen) in 200 µl of DMEM for 20 min, and were then added to the wells in a final volume of 0.75 ml with serum-free DMEM. Eight hours later, HEK-29T cells were cotransfected with HIV-1 Gag or RT DNA using the Lipofectamine 2000 reagent (Invitrogen), following the protocol set by the manufacturer. An additional 1.00 ml of DMEM containing 20% FCS was also added to the cells at this time. Cells were then cultured and assayed for the appropriate activity at 48 h post transfection.

2.4 Results

2.5.1 Interaction of HIV-1 Pol with cellular LysRS.

We first determined by coimmunoprecipitation if Pol or Pol fragments expressed in the cell would interact with endogenous LysRS. 293T cells were transfected with a plasmid coding for HIV-1 Pol or fragments of Pol, all C-terminally tagged with V5. 48 hrs post-transfection, endogenous LysRS was immuno-precipitated from cell lysate with anti-LysRS. Western blots of the immunoprecipitate were then probed with anti-V5 to examine the ability of endogenous LysRS to interact with Pol or Pol fragments, and these results are shown in Figure 2.1. Panel A shows a cartoon of the Pol and Pol fragments tested, and displays the coding domains for protease (PR), RT, and integrase (IN), and the position of the various structural domains of RT, that include the finger-palm domain (FP), TH, connection domain (CD), and the RNAseH domain (RN); Panel B shows the cellular expression of these fragments, while panel C shows the ability of the fragments to be coimmunoprecipitated with anti-LysRS. In the left blot of panel C, it can be seen that Pol binds to LysRS, but weakly, and removal of p6* and PR does not alter this weak binding. However, removal of N-terminal sequences in Pol through FP result in a significant increase in binding, which is retained when TH is also removed. In the middle blot, it can be seen that the C-terminal deletion of IN in Pol also results in an increased binding to LysRS compared to full-length Pol. The right blot in panel C shows that the N-TH fragment binds much more poorly to LysRS than the N-RN fragment, but because cellular expression of N-CD is poor, the results for this fragment are non-informative.

The data in Figure 2.1 indicate that sequences in Pol binding to LysRS are found

within CD/RN of RT. They also demonstrate that removal of either N-terminal sequences through FP, or the C-terminal IN results in significant increases in binding to LysRS. The much weaker binding of LysRS to Pol, PR-C, and FP-C may reflect a conformation that masks the LysRS binding sequences within Pol, but an alternative explanation is that this much weaker binding represents non-specific background binding, which would also account for the similar weak binding exhibited by the N-TH fragment. In the natural state, where Pol is covalently attached in *cis* to Gag, the LysRS binding sites in Pol (CD/RN) may be more exposed.

2.5.2 Interaction of HIV-1 RT with cellular LysRS.

Finer mapping of the LysRS binding site in Pol RT sequences was done by measuring the cytoplasmic interaction of wild type and mutant forms of RT with endogenous LysRS. 293T cells were transfected with a plasmid coding for V5-tagged RT, and the ability of RT to coimmunoprecipitate with cellular LysRS was examined by immunoprecipitating cell lysate with anti-LysRS, and probing Western blots of the immunoprecipitate with anti-V5. To test for the specificity of the interaction, the presence in the immunoprecipitate of two common cellular proteins, GAPDH and β-actin, were also probed for with antibodies to these proteins. The results, shown in Figure 2.2A, indicate an interaction between RT and LysRS, but no interaction between LysRS and either GAPDH or β-actin. In panel B, we tested if RNA plays a role in facilitating the LysRS/RT interaction by treating the anti-LysRS immunoprecipitate after bead washing with RNAses A and T1. The results surprisingly show that removal of RNA results in a stronger interaction between LysRS and RT, suggesting that RNA in the immunoprecipitate may be partially blocking the interaction between anti-V5 and the

V5 epitope. Similar results are seen if cell lysate is treated with RNase prior to immunoprecipitation (data not shown), and the results indicate that RNA does not play a role in facilitating the LysRS/RT interaction.

We next mapped the sequences in RT that are required for interacting with LysRS. 293T cells were transfected with plasmids coding for RT or RT fragments, V5-tagged at the C-terminus. Cell lysates were immunoprecipitated with anti-LysRS, and Western blots were probed with anti-V5 to determine which RT fragments immunoprecipitated with LysRS. The results are shown in Figure 2.2,C-E. Figure 2.2C displays a cartoon of the RT fragments used, Figure 2.2D shows their cellular expression, and Figure 2.2E shows the ability of the RT fragments to be coimmunoprecipitated with anti-LysRS. As shown in the left gel in panel E, there is an increasing ability of LysRS to bind to RT fragments in the order RT< FP-CD< TH-RN, while the much weaker binding of FP-TH is suggestive of non-specific background binding. It can also be seen in panel D that because the CD-RN and TH-CD fragments are expressed very poorly, the binding results for these fragments are non-informative. The results, therefore, from Figures 2.1 and 2.2 indicate a binding site for LysRS mapping to the RT CD/RN domains in Pol. While the interaction between Gag and LysRS has been demonstrated both *in vivo* (274) and *in vitro* (307), attempts to demonstrate an *in vitro* interaction between either purified RT or purified TH-RN and purified LysRS, using coimmunoprecipitation with anti-LysRS, have thus far failed (data not shown). Furthermore, the addition of either purified RT or TH-RN produced in *E. coli* to a cell lysate, followed by immunoprecipitation of endogenous LysRS with anti-LysRS also failed to show an interaction with LysRS (data not shown). Thus, we are

only able to show an interaction between LysRS and RT or TH-RN when both are expressed in the cell (see Figure 2.2C). This could indicate a requirement for unknown cell factors whose compartmentalization is disrupted in cell lysate, but could also be indicative of differences in modification between *E.coli*-produced RT fragments (including the presence of a His-tag used for purification) and RT fragments synthesized in mammalian cells. Also, since the source of viral LysRS is newly-synthesized LysRS (213), a requirement for the LysRS/RT interaction may also be the use of newly-synthesized RT.

2.5.3 The LysRS/Pol interaction is not required for Gag/Pol interaction.

We next investigated whether the LysRS/Pol interaction was essential for the interaction between Gag and Pol. We have previously shown that in 293T cells expressing Gag and Pol from separate plasmids, Pol was incorporated into Gag VLPs, and was itself sufficient for facilitating the selective packaging of $tRNA^{Lys}$ (72). That work also showed that removal of sequences either upstream or downstream of RT-coding sequences did not reduce incorporation of the truncated Pol, suggesting that sequences within RT were responsible for Pol incorporation into Gag VLPs.

In Figure 2.3, we provide additional evidence for a cytoplasmic interaction between Gag and Pol, and one that relies upon the RT sequences within Pol. Figure 2.3A shows the protein coding regions of full-length Gag and a truncated Gag, GagΔp6, which is missing the C-terminal p6 sequences. In Figure 2.3B, Pol was immunoprecipitated with anti-V5 from lysates of cells expressing both V5-tagged Pol and either Gag or GagΔp6, and determined if Gag was detected on western blots of the immunoprecipitate

probed with anti-CA. These results demonstrate a cytoplasmic interaction between Gag and Pol, and also show a requirement for Gag p6 sequences for this reaction, a finding supported by a previous report demonstrating a requirement for p6 sequences for the incorporation of Pol into mutant Gag VLPs, in which the NC and sp2 sequences in Gag had been replaced by a yeast leucine zipper (72).

In Figure 2.3C, the role of RT in the Gag/Pol interaction is demonstrated by detecting a cytoplasmic interaction between Gag and RT. Gag was immunoprecipitated with anti-CA from lysates of cells also expressing RT, and RT was detected in western blots of the immunoprecipitate with anti-RT. The data in panel D also shows the ability of HIV-1 RT to be incorporated into HIV-1 Gag VLPs, but not into RSV Gag VLPs. In order to directly compare the Gag VLPs made from two different species, HIV-1 Gag was expressed from viral mRNA rather than from codon-optimized mRNA (as for Figure 2.3B, C). 293T cells were cotransfected with a plasmid coding for HIV-1 RT, and a plasmid coding for either protease-negative RSV Gag or GagFS-, a plasmid coding for all HIV-1 viral proteins except GagPol (341). Western blots of viral lysates show the incorporation of RT into the viruses. Also shown is the fact that when 293T cells are cotransfected with plasmids coding for Rous sarcoma virus (RSV) Gag and HIV-1 RT, RT is not found incorporated into the RSV Gag VLPs.

In Figure 2.4, we determined whether the LysRS/RT interaction was required for the cytoplasmic interaction between Gag and RT, or for the incorporation of Pol into Gag VLPs, by examining the effect of overexpression or underexpression of LysRS upon these parameters. 293T cells were cotransfected with plasmids coding for HIV-1 Gag and RT. In addition, overexpression of LysRS was achieved by also transfecting

cells with a plasmid coding for LysRS, while underexpression of LysRS was obtained using siRNA to LysRS. Under the conditions used, overexpression of LysRS in the cytoplasm results in a modest 2 fold increase in the incorporation of LysRS into virions (68). However, under the conditions used here for siRNA transfection, we have reported that siRNA to LysRS results in an 80% decrease in LysRS incorporated into the virions (213). Assuming that the incorporation of LysRS into virions reflects its presence in the $tRNA^{Lys}$ packaging/annealing complex, the results in Figure 2.4A indicate that the alterations in the concentration of LysRS in the complex resulting from over-or under-expression of LysRS had no effect upon the interaction of Gag with RT.

In Figure 2.4B, we tested the effect of LysRS reduction upon the ability of Pol to be incorporated into Gag VLPs. 293T cells were triply transfected with siRNA to LysRS, and plasmids coding for Gag (GagFS-) and Pol. As shown in panel B, the reduction of cellular LysRS had no effect upon the ability of Pol to be incorporated into Gag VLPs. These results indicate that the interaction between LysRS and Pol sequences, which might form a Gag/LysRS/Pol bridge, is not essential for facilitating the observed Gag/Pol or Gag/RT interactions.

2.5 Discussion

While $tRNA^{Lys}$ is targeted for incorporation into HIV-1 by a select interaction of HIV-1 Gag with LysRS, RT sequences within GagPol must also be present, or LysRS will be packaged without $tRNA^{Lys}$ (70, 296). A model of how $tRNA^{Lys3}$ incorporation might occur, via the formation of a $tRNA^{Lys}$ packaging/annealing complex, is shown in Figure 2.5A, in which a Gag/GagPol/viral RNA complex interacts with a $tRNA^{Lys3}$/LysRS complex, with Gag binding specifically to LysRS, and $tRNA^{Lys3}$ binding to TH in the RT sequence in GagPol. $tRNA^{Lys3}$ is uncharged in the virus (257), and must be so if it is to act as a primer for reverse transcriptase. It is not known if $tRNA^{Lys3}$ is uncharged at the time of these interactions, or if the interaction of Gag with LysRS causes both deacylation and the release of deacylated $tRNA^{Lys3}$. The Gag/GagPol ratio in the $tRNA^{Lys}$ packaging/annealing complex is lower than will be found in the budding virion, based on the following data. When transfected 293T cells producing HIV-1 are pulsed for 10 minutes with ^{35}S-Cys/Met, ~15% of newly-synthesized Gag and >95% of newly-synthesized GagPol are found associated with membrane domains enriched in lipid rafts (222), which is characteristic of membrane from which HIV-1 buds (405, 416). The Gag and GagPol molecules at this membrane domain are presumably in a complex since the movement of GagPol to membrane requires association with Gag, and this Gag/GagPol association appears to be driven by the interaction between homologous Gag sequences within Gag and GagPol (430, 506). Since the ratio of synthesis of Gag/GagPol in cells has been estimated to be approximately 20:1 (193), the ratio of newly-synthesized Gag:GagPol at lipid raft domains would be 3:1, i.e., much lower than the Gag:GagPol ratio found in immature

virions. With current estimates of the number of Gag molecules in an immature HIV-1 being ~4000 (42), the 20:1 synthesis ratio of Gag:GagPol may suggest approximately 100 molecules of GagPol per virion, or in the $tRNA^{Lys}$ packaging/annealing complex, ~300 molecules Gag: 100 molecules GagPol. It was also observed that during a 30 minute chase period, more Gag molecules moved towards lipid raft-enriched membrane (222), implying that the $tRNA^{Lys}$ packaging/annealing complex may represent a very early assembly intermediate to which more Gag is later added.

In the model in Figure 2.5A, GagPol is shown as a dimer because dimerization of PR will be required to activate this enzyme for later Gag and GagPol processing. LysRS is shown as a monomer, since the dimerization site in LysRS includes the binding site for Gag, and monomeric LysRS has been shown to interact with Gag *in vitro* (307).

The data in Figure 2.2B indicate that RNA does not play a role in the LysRS/RT interaction. Similarly, we have found that while RNA appears to be required for the Gag/GagPol interaction, it is not for a Gag/Pol interaction (72). Thus, a direct need for RNA in the Gag/GagPol interaction is implied by the fact that replacement of Gag NC with a yeast leucine zipper motif allows for Gag particle formation without incorporation of either general RNA or GagPol. However, these same mutant Gag VLPs do package Pol (72).

The actual structural conformation of GagPol that would allow for the Pol sequences to fold backwards and interact with Gag is not known. While the association of GagPol with Gag is most likely driven by an interaction between homologous sequences in Gag and GagPol, we have used the simple cartoon in Figure 2.5B to point out how the Pol interaction with Gag could prove useful to the virus. The model shows

tRNA^{Lys3} bound to TH in RT, which is based both on *in vitro* studies on the interaction of purified HIV-1 RT with tRNA^{Lys3} (14, 134), and *in vivo* studies on the effect of C-terminal deletions in GagPol upon tRNA^{Lys3} incorporation into HIV-1 (71, 296). The model also shows the 5′ region of viral genomic RNA. HIV-1 genomic RNA is packaged into the virus through interactions between nucleocapsid protein sequences in Gag and specific stem/loop structures at the 5′ end of the genomic RNA (27, 189), and the primer binding site in viral RNA is located within 100 nucleotides upstream of these sequences. Thus, the movement of Pol back towards Gag might facilitate transfer of tRNA^{Lys3} from LysRS to RT by an unknown mechanism, and place tRNA^{Lys3} closer to the PBS region in viral RNA where annealing is to occur. Also, during viral maturation, the first protease cleavage is between NCp7 and SP1 (441), and may be initiated before viral budding is complete. An interaction between Pol and Gag could insure that Pol is retained in the partially closed budding particle. The fact that p6 is required for the interaction of Gag with Pol (Figure 2.3B, C) is of interest in this context since it has been reported that the deletion of p6 from Gag resulted in a lower concentration of Pol products in protease-positive HIV-1, but no change in GagPol incorporation in protease-negative viruses (582).

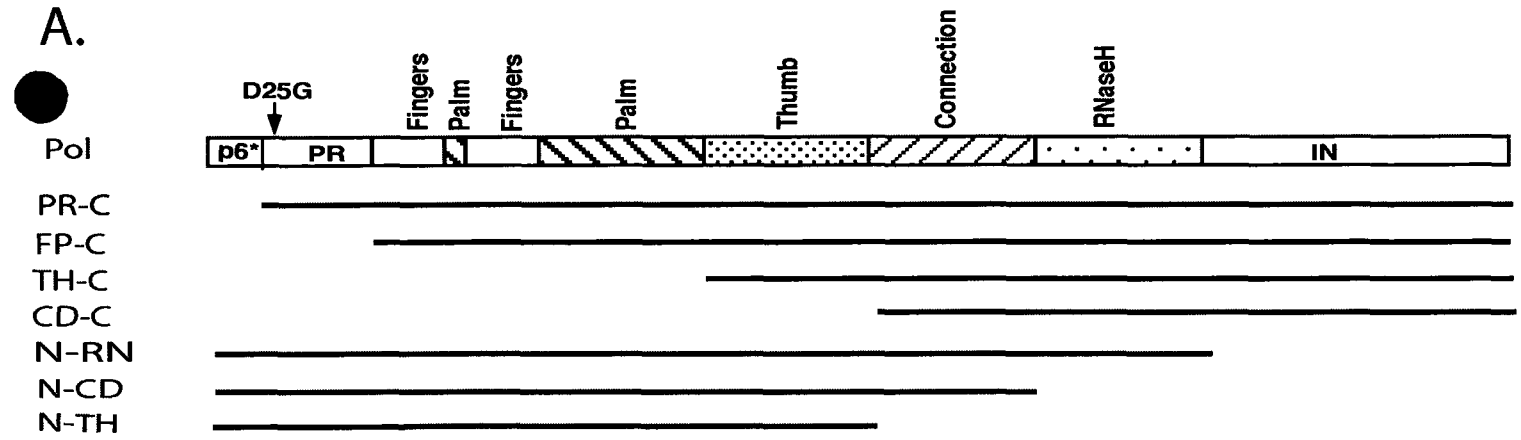
The model in Figure 2.5B shows the dual interaction of LysRS with both the C-terminal domain of Gag capsid and the RT connection domain in Pol. Determination of the site in LysRS for binding RT has proven to be technically difficult, and is not yet known. The data in Figure 2.4, however, indicates that the LysRS/RT interaction is not required for the interaction between Gag and Pol or RT, and other interactions, involving matrix and p6 sequences in Gag and RT TH sequences in Pol, have been reported (342).

Since C-terminal deletions of GagPol that include the RT connection domain do not inhibit $tRNA^{Lys3}$ packaging (71), it is clear that the LysRS/RT interaction is also not involved in facilitating $tRNA^{Lys3}$ packaging. On the other hand, that same report showed that the CD in RT is required for $tRNA^{Lys3}$ annealing, i.e. virions in which GagPol is C-terminally deleted through the RT CD can still selectively package $tRNA^{Lys3}$, but don't anneal it to the viral RNA genome. This could mean that a Gag/LysRS/RT interaction may be involved in conformation changes facilitating movement of $tRNA^{Lys3}$ towards the primer binding site on the viral RNA. The annealing of $tRNA^{Lys3}$ to viral RNA may not only involve Gag NC and $tRNA^{Lys3}$, but RT as well, and a Gag/LysRS/RT interaction may bring a $tRNA^{Lys3}$/RT complex to the primer binding site where, once $tRNA^{Lys3}$ is annealed by NC, the associated, processed, RT is ready to initiate reverse transcription.

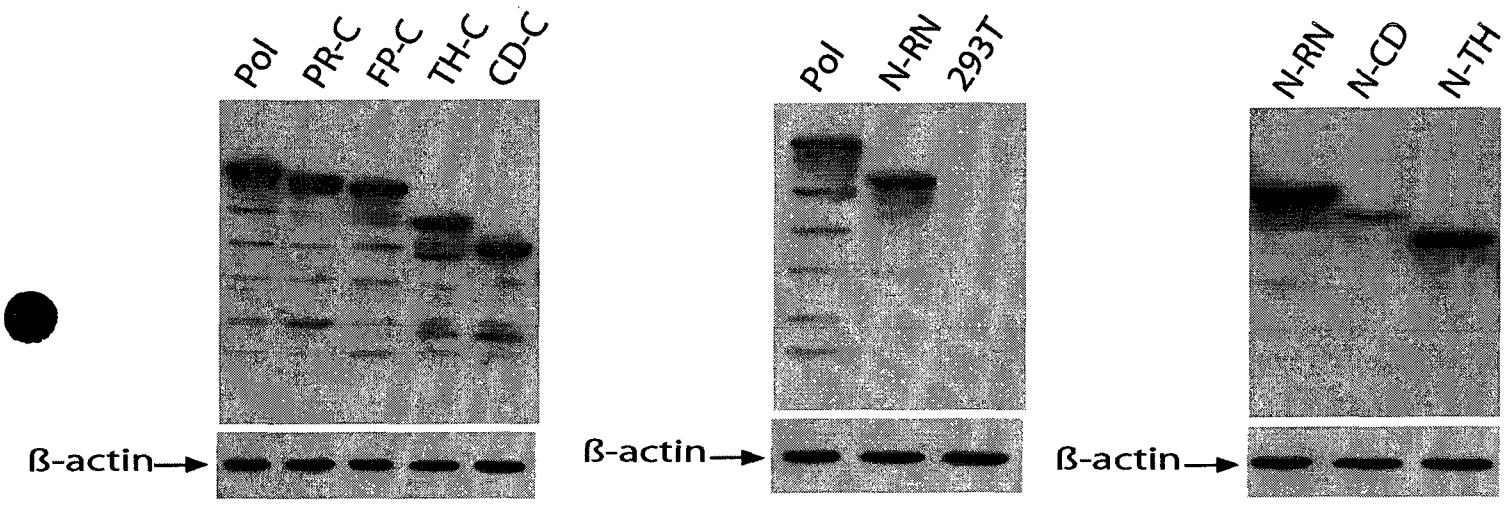
Figure 2.1 Interaction of Pol sequences with LysRS.

293T cells were transfected with plasmids coding for V5-tagged full-length Pol or truncated Pol fragments. Interaction of these Pol fragments with endogenous LysRS was determined by immunoprecipitation of cell lysates with anti-LysRS, followed by probing western blots of the anti-LysRS immunoprecipitates with anti-V5. **(A)** Cartoon of Pol and Pol fragments expressed in 293T cells. **(B)** Cellular expression of Pol fragments. Western blots of cellular lysates were probed with anti-V5 (upper panel) or anti-β-actin (lower panel). **(C)** Interaction of LysRS with Pol fragments. Cell lysates were immunoprecipitated with anti-LysRS, and western blots of the immunoprecipitates were probed with anti-V5.

A.



B. **W.B. (Top) anti-V5, (Bottom) anti- ß-actin**



C. **I.P. anti-LysRS, W.B. anti-V5**

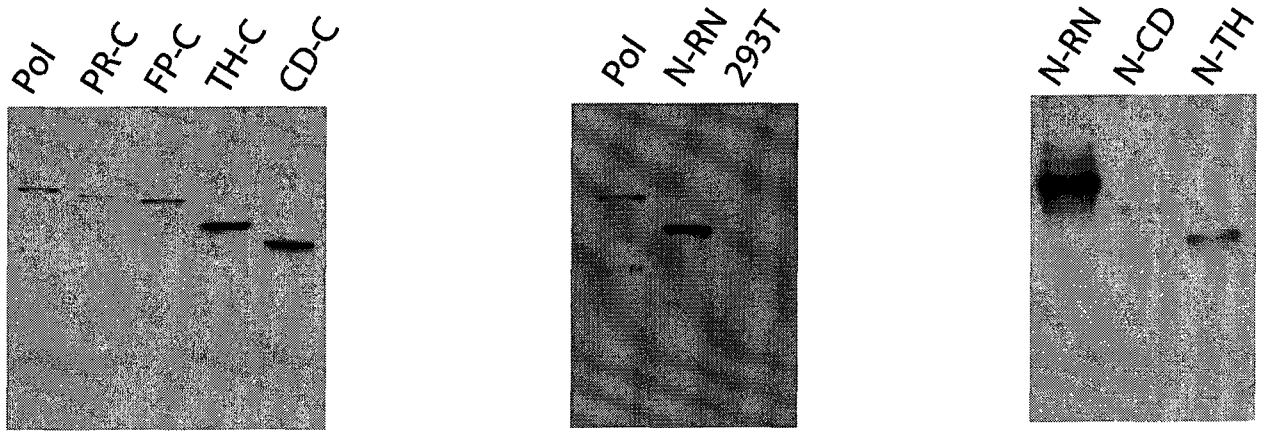
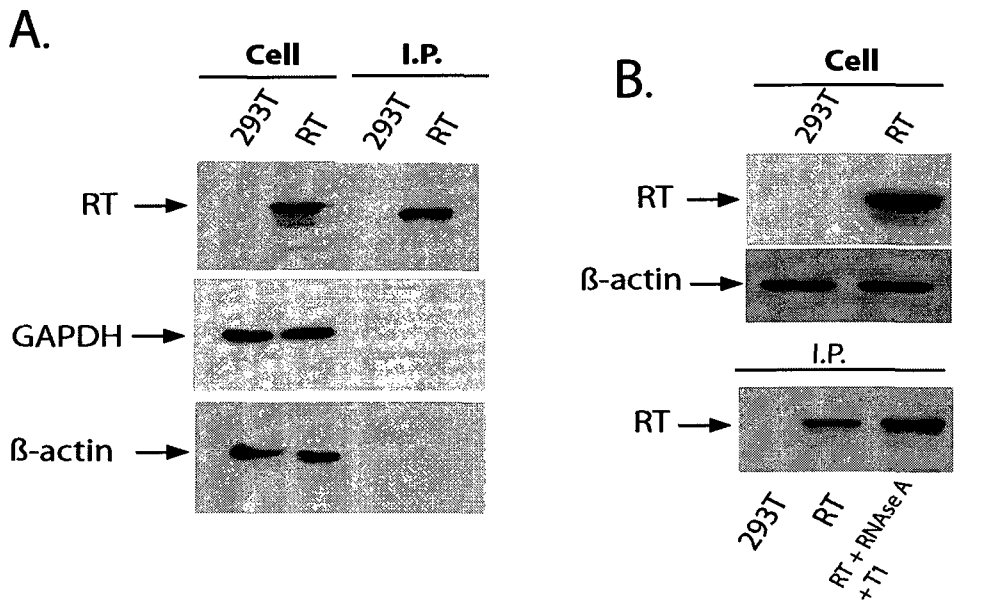


Figure 2.2 Interaction of RT sequences with LysRS.

293T cells were transfected with plasmids coding for V5-tagged full-length RT or truncated RT fragments. Interaction of these RT fragments with endogenous LysRS was determined by immunoprecipitation of cell lysates with anti-LysRS, followed by probing western blots of the anti-LysRS immunoprecipitates with anti-V5. **(A)** Specificity of RT and LysRS interaction. 293T cells were transfected with a plasmid coding for full-length RT. Western blots of cell lysates were probed with anti-V5 (left upper panel), anti-GAPDH (left middle panel) or anti-β-actin (left lower panel). Cell lysates were then immunoprecipitated with anti-LysRS, and western blots of the immunoprecipitates were probed with anti-V5 (right upper panel), anti-GAPDH (glyceraldehyde-3-phosphate dehyrogenase (right middle panel)) or anti-β-actin (right lower panel). **(B)** RNA-independence of the RT/LysRS interaction. Lysates of cells expressing RT and β-actin (western blots shown in the upper two panels) were immunoprecipitated with anti-LysRS. The immunoprecipitate was, or was not, treated with RNase A and T1 RNase, and the bottom panel shows a western blot of untreated or treated immunoprecipitate probed with anti-V5. **(C)** Cartoon of RT and RT fragments expressed in 293T cells. **(D)** Cellular expression of RT fragments. Western blots of cellular lysates were probed with anti-V5 (upper panel) or anti-β-actin (lower panel). **(E)** Interaction of LysRS with RT fragments. Cell lysates were immunoprecipitated with anti-LysRS, and western blots of the immunoprecipitates were probed with anti-V5.


A.
Cell
I.P.
293T
RT
293T
RT
RT
GAPDH
ß-actin
B.
Cell
293T
RT
RT
ß-actin
I.P.
RT
293T
RT
RT + RNAse A + T1

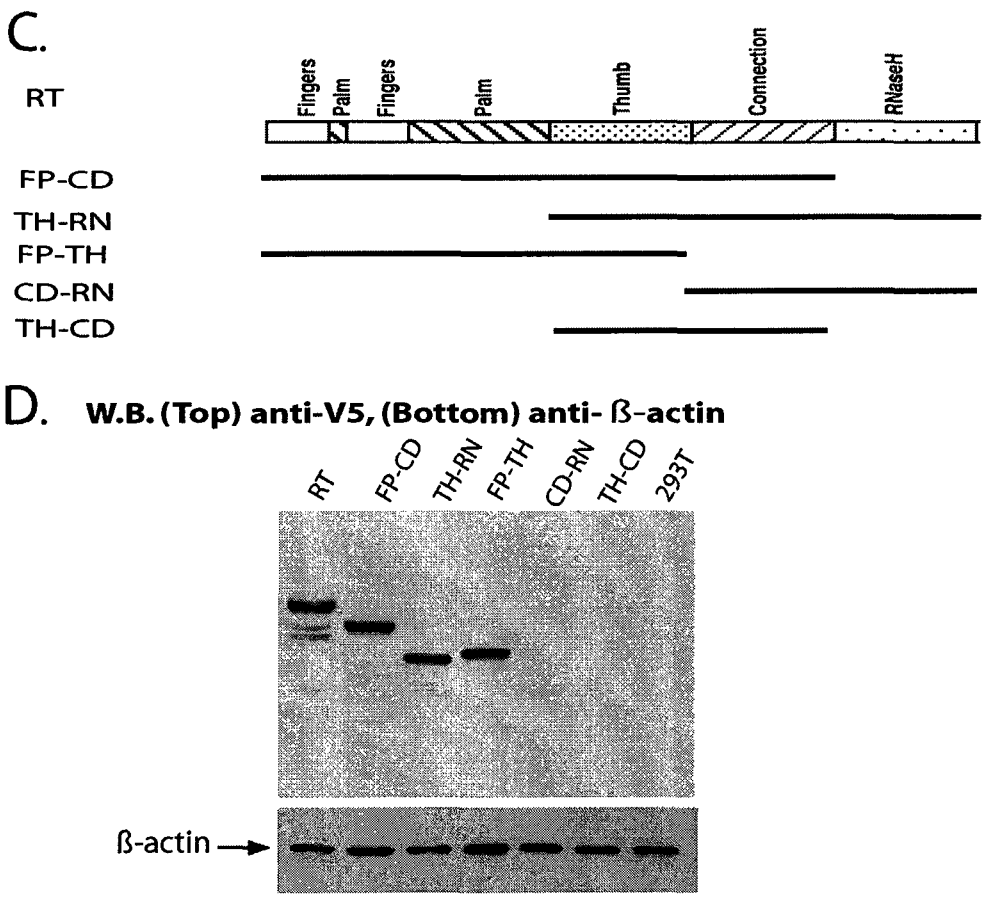
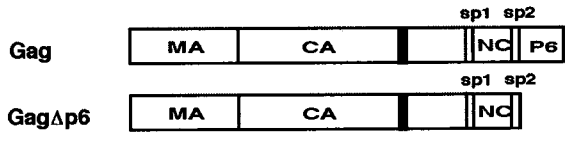

C.
RT
Fingers
Palm
Fingers
Palm
Thumb
Connection
RNaseH
FP-CD
TH-RN
FP-TH
CD-RN
TH-CD
D. W.B. (Top) anti-V5, (Bottom) anti- ß-actin
RT
FP-CD
TH-RN
FP-TH
CD-RN
TH-CD
293T
ß-actin
E I.P. anti-LysRS, W.B. anti-V5
RT
FP-CD
TH-RN
FP-TH
CD-RN
TH-CD
293T

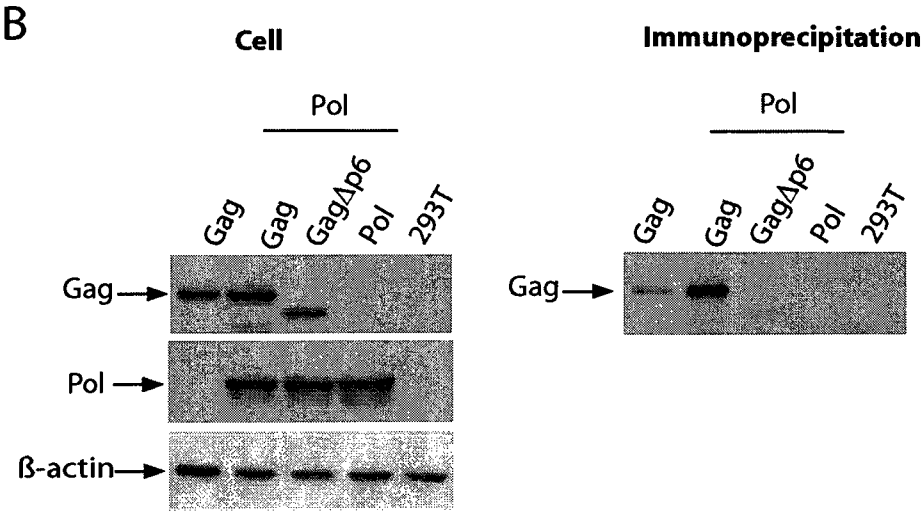
Figure 2.3 Pol or RT interaction with Gag.

(A-C). 293T cells were cotransfected with a plasmid coding for Gag or GagΔp6, and a plasmid coding for either V5-tagged Pol (B) or RT (C). **(A)** Coding regions of Gag and GagΔp6. **(B)** Left panel: Cellular expression of Gag, GagΔp6, Pol, and β-actin, using western blots of cellular lysates probed, respectively, with anti-CA (upper panel), anti-V5 (middle panel), or anti-β-actin (lower panel). Right panel: Cell lysates were immunoprecipitated with anti-V5, and western blots of the immunoprecipitates were probed with anti-CA. **(C)** Left panel: Cellular expression of RT, Gag, and β-actin, using western blots of cellular lysates probed, respectively, with anti-RT (upper panel), anti-CA (middle panel) or anti-β-actin (lower panel). Right panel: Cell lysates were immunoprecipitated with anti-CA, and western blots of the immunoprecipitates were probed with anti-RT. **(D)** 293T cells were cotransfected with a plasmid coding for HIV-1 RT and either GagFS- (coding for all HIV-1 proteins except GagPol), or a plasmid coding for RSV Gag.P-. Left panel: Cellular expression of RT and β-actin, using western blots of cellular lysates probed, respectively, with anti-V5 (left upper panel) or anti-β-actin (left lower panel). Right panel: Western blots of lysates of extracellular Gag VLPs, probed with anti-V5 (right upper panel) or anti-CA (right middle panel). Western blots of RSV VLPs, probed with RSV anti-CA (right lower panel).

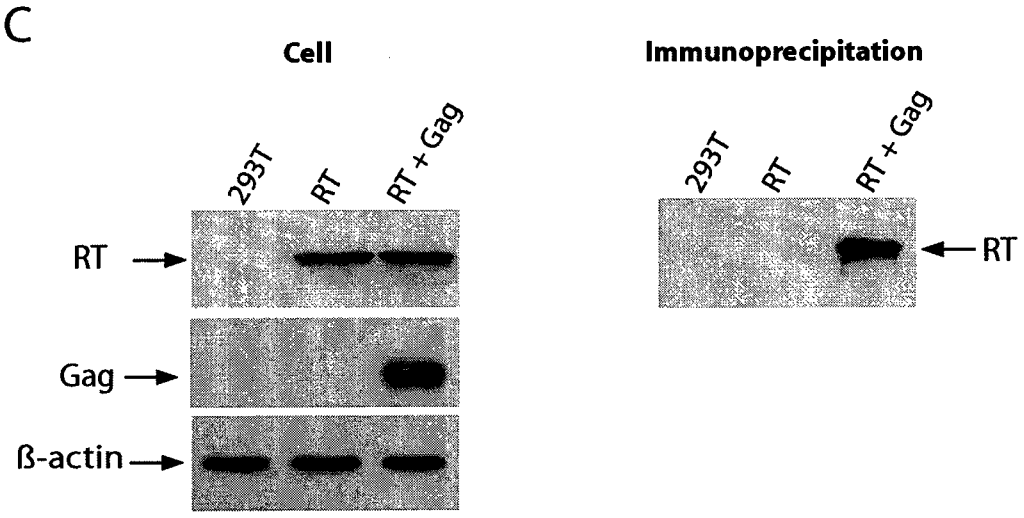
A.


sp1 sp2
Gag
MA
CA
NC
P6
sp1 sp2
GagΔp6
MA
CA
NC

B


Cell
Immunoprecipitation
Pol
Pol
Gag
Gag
GagΔp6
Pol
293T
Gag
Gag
GagΔp6
Pol
293T
Gag
Gag
Pol
ß-actin

C


Cell
Immunoprecipitation
293T
RT
RT + Gag
293T
RT
RT + Gag
RT
Gag
ß-actin
RT

D

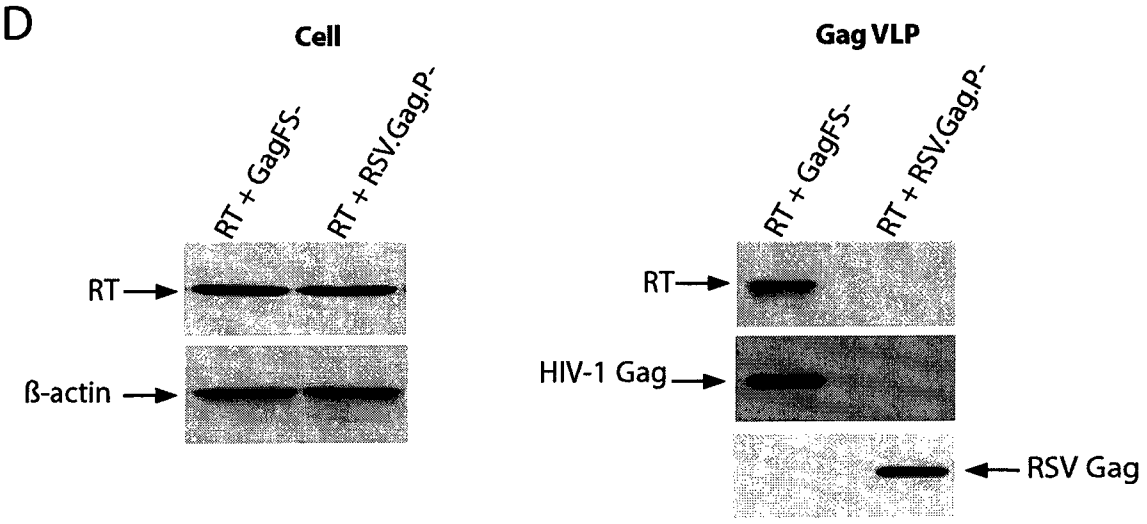
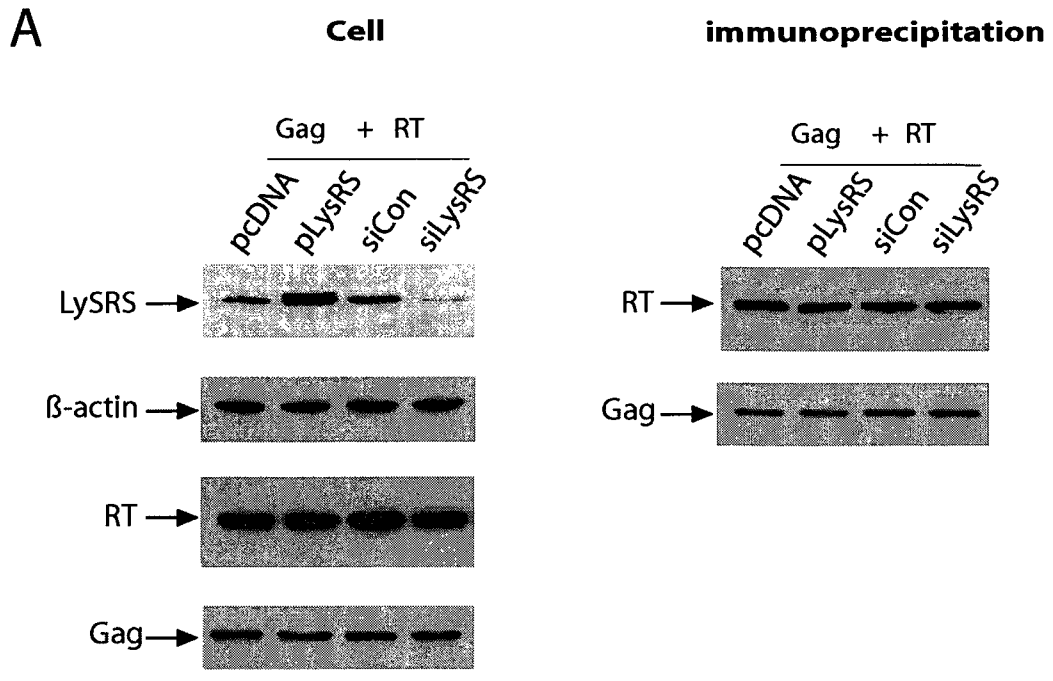

Cell
Gag VLP
RT + GagFS-
RT + RSV.Gag.P-
RT + GagFS-
RT + RSV.Gag.P-
RT
ß-actin
RT
HIV-1 Gag
RSV Gag

Figure 2.4 Effect of alterations in LysRS expression upon Gag interaction with Pol or RT.

(A) 293T cells were cotransfected with plasmids coding for Gag and RT. Some cells were also transfected with a third plasmid, either empty (pcDNA), or coding for LysRS (pLysRS), while other cells were transfected with siRNA to either luciferase (sicon) or to LysRS (siLysRS), 8 hours prior to transfection with DNA plasmids for Gag and RT. In panel A, left, cell expression of proteins 48 hours post-transfection is shown by probing western blots of cell lysates with either anti-LysRS (left upper panel), anti- β-actin (left second panel), anti-RT (left third panel), and anti CA (left lower panel). The cell lysates were immunoprecipitated with anti-CA, and western blots of the immunoprecipitate (right side, panel (A) were probed with either anti-RT (right upper panel) or anti-CA (right lower panel). **(B)** Eight hours after transfecting 293T cells with siCon or siLysRS, the cells were cotransfected with GagFS- and a plasmid coding for Pol. In panel B, left, cell expression of proteins 48 hours post-transfection is shown by probing western blots of cell lysates with either anti-LysRS (left upper panel), anti- β-actin (left second panel), anti-V5 (left third panel), and anti CA (left lower panel). Right panel: Western blots of lysates of extracellular Gag VLPs, probed with anti-V5 (right upper panel) or anti-CA (right lower panel).


A
Cell
immunoprecipitation
Gag + RT
pcDNA
pLysRS
siCon
siLysRS
LySRS
ß-actin
RT
Gag
Gag + RT
pcDNA
pLysRS
siCon
siLysRS
RT
Gag

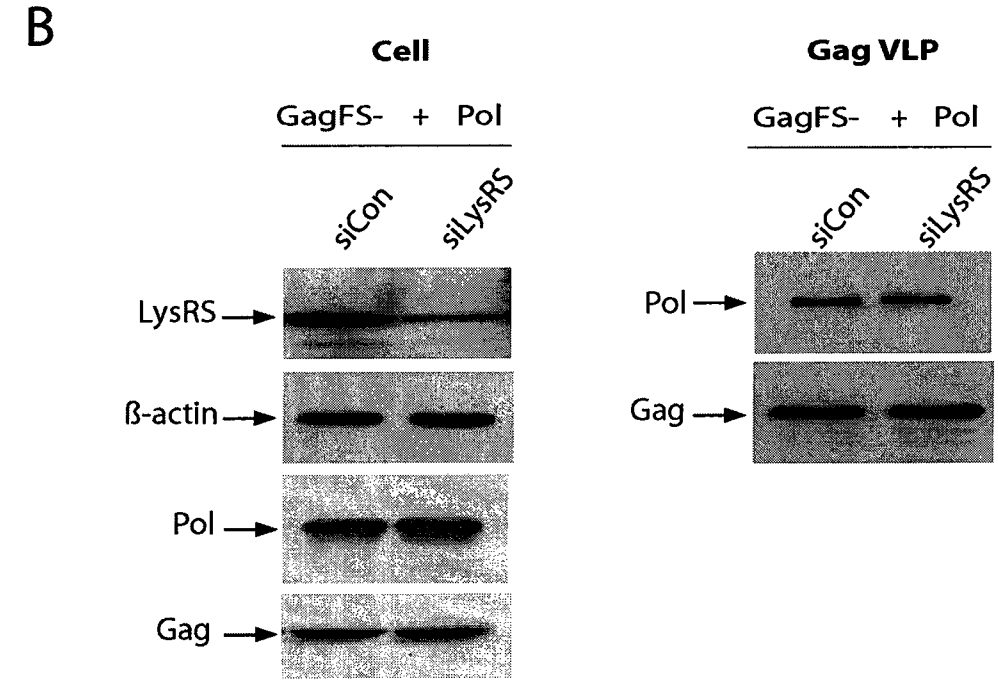
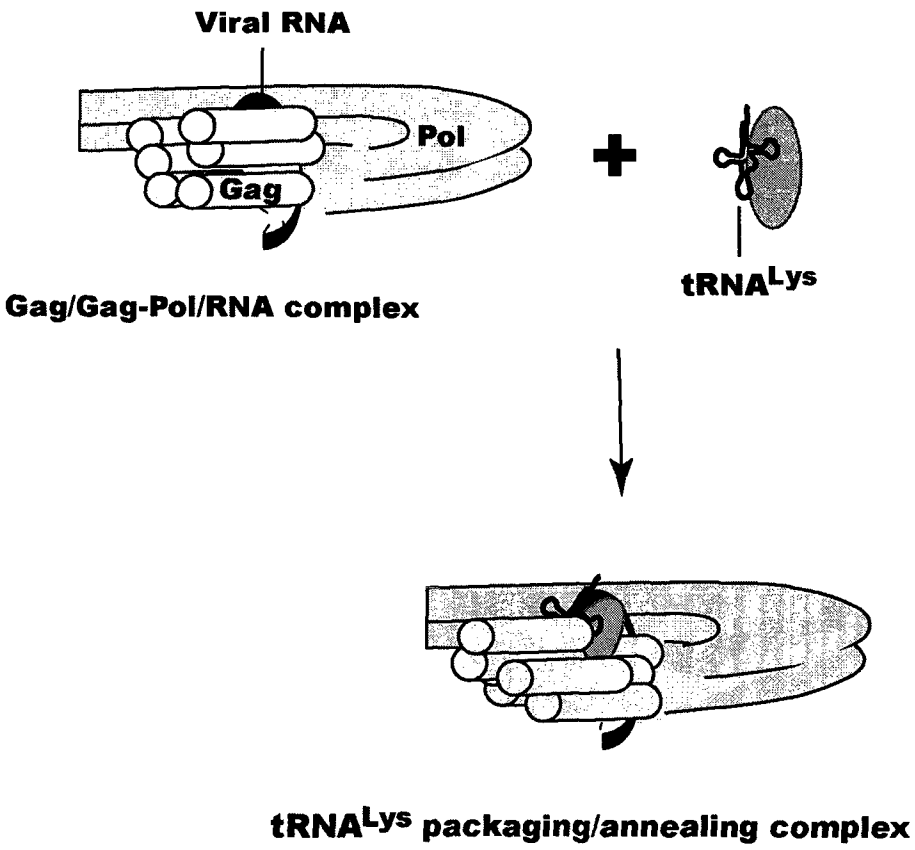

B
Cell
Gag VLP
GagFS- + Pol
siCon
siLysRS
LysRS
ß-actin
Pol
Gag
GagFS- + Pol
siCon
siLysRS
Pol
Gag

Figure 2.5 The $tRNA^{Lys}$ packaging/annealing complex.

(A) Formation of the $tRNA^{Lys}$ packaging/annealing complex. The picture shows a previously formed Gag/GagPol/viral RNA complex interacting with a $tRNA^{Lys3}$/LysRS complex, with Gag interacting with both GagPol and LysRS, and GagPol also interacting with $tRNA^{Lys}$. The facts supporting this picture are described in detail in the text. **(B)** Proposed relationships existing between components of the $tRNA^{Lys}$ packaging/annealing complex. Although the actual conformation (s) of GagPol is not known, data in this paper and elsewhere suggests an interaction between Pol and Gag sequences. As described in the text, the nature of this interaction is not clear, and while this work indicates the occurrence of a Gag/LysRS/Pol interaction, it appears that other interactions also occur. As described in the text, we propose that these relationships will facilitate $tRNA^{Lys3}$ packaging and annealing, as well as facilitate retention of partially processed Pol during early stages of budding.

A


Viral RNA
Pol
Gag
Gag/Gag-Pol/RNA complex
tRNA^Lys
tRNA^Lys packaging/annealing complex

B

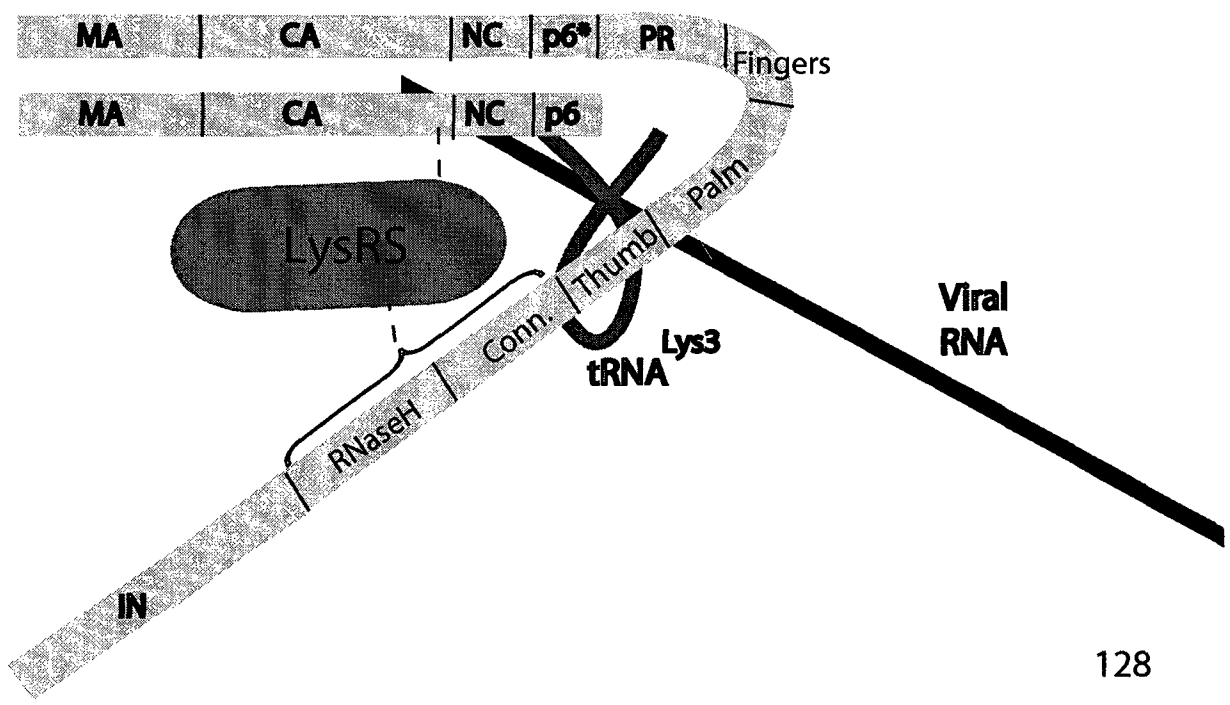

MA
CA
NC
p6*
PR
Fingers
MA
CA
NC
p6
LysRS
Palm
Thumb
Conn.
RNaseH
IN
tRNA Lys3
Viral RNA

Table 2.1 Plasmids used in this study.

Plasmid[a]	Codon Usage	Protein expressed
GagFS-	Virus	Gag
GagΔp6	Codon optimized	Gag missing *p6*
RSV.Gag.P-	Virus	RSV Gag
Gag	Codon optimized	Gag
Pol	Codon optimized	Pol
RT	Codon optimized	RT
pLysRS	Human	LysRS

[a] All viral proteases are inactive.

CHAPTER III

Roles of Gag and mature nucleocapsid in facilitating the $tRNA^{Lys3}$ annealing and initiation of reverse transcription in HIV-1.

(This chapter was adapted from a manuscript submitted (2008))

3.1 Preface

The name of the $tRNA^{Lys}$ packaging/annealing complex shown in Figure 1.10 indicates that we think this complex is involved in both the packaging and annealing of $tRNA^{Lys3}$ to the viral RNA genome. But while both Gag-NC and mature NCp7 are believed to play roles in the annealing of $tRNA^{Lys3}$, only Gag is present in this complex composed of precursor proteins. In Chapter 3, presenting a submitted manuscript, we address the issue of what contributions both Gag and NCp7 play in the annealing of $tRNA^{Lys3}$. We show that annealing by Gag results in the suboptimal placement of $tRNA^{Lys3}$ onto the viral RNA, using both a primer extension assay and a PBS-occupancy assay. We then make use of our discovery that APOBEC3G (A3G) can inhibit the annealing of $tRNA^{Lys3}$ by NCp7, but not the annealing of $tRNA^{Lys3}$ by Gag, to explore whether $tRNA^{Lys3}$ is first annealed loosely by Gag, followed by a fine tuning by NCp7. Our studies with A3G indicate a more complicated story, suggesting that NCp7 annealing of $tRNA^{Lys3}$ occurs either in the absence of Gag-annealing in protease-positive viruses, or that $tRNA^{Lys3}$ annealed by Gag is "reannealed" by NCp7.

A3G has been shown to be able to inhibit $tRNA^{Lys3}$ annealing to the HIV-1 genome both *in vivo* and *in vitro*, independently of enzyme activity, and this inhibition requires interaction of A3G with NCp7. Because the number of A3G molecules in each virion are low (7 +/- 4) compared to the number of NCp7 molecules in a mature virion (approx. 1800), it is not clear how such a small number of A3G molecules can accomplish this job independently of enzyme activity. We have hypothesized that A3G may bind to molecules involved in the $tRNA^{Lys}$ packaging/annealing complex, which would bring A3G closer to the region of annealing. In the addendum to Chapter 3, we

present our preliminary studies showing that A3G interacts with one such molecule, LysRS.

3.2 Abstract

We have measured the initiation of reverse transcription from viral RNA isolated from both protease-positive (Pr+) and protease-negative (Pr-) viruses, and also measured $tRNA^{Lys3}$ annealing in these viral RNAs by determining the occupancy of the primer binding site by $tRNA^{Lys3}$. While initiation of reverse transcription in the presence of excess deoxynucleotide triphosphates is reduced approximately 25% when initiated from Gag-annealed $tRNA^{Lys3}$ compared to mature nucleocapsid (NCp7)-annealed $tRNA^{Lys3}$, Gag-annealed $tRNA^{Lys3}$ binds more weakly to viral RNA, as indicated by its weaker (73% reduction) ability to incorporate sub-optimal amounts of the first dNTP, dCTP, and by its more ready displacement from the primer binding site (PBS) by DNA synthesized from a downstream primer. APOBEC3G (A3G) was also found to inhibit both initiation of reverse transcription and annealing of $tRNA^{Lys3}$, but only in Pr+ virions, and not in Pr- virions. Thus, in Pr+ viruses, resistance of Gag-annealed $tRNA^{Lys3}$ to A3G-inhibition of reverse transcription is overcome by the natural exposure of viral RNA to NCp7. These results are further supported by differences found in the ability of purified GagΔp6 or NCp7 to anneal $tRNA^{Lys3}$ *in vitro* to viral RNA, in the presence or absence of A3G. *In vitro* studies measured both initiation of reverse transcription (+6 and +1 nucleotide extension of $tRNA^{Lys3}$), and annealing (PBS occupancy and measurements of ^{32}P-$tRNA^{Lys3}$ annealed to viral RNA).

3.3 Introduction

In HIV-1, minus-strand viral DNA synthesis by reverse transcriptase is initiated by a cellular tRNA, $tRNA^{Lys3}$, which binds to sequences in the 5′ region of the viral RNA genome (302). These sequences include the primer binding site (PBS), an 18 nucleotide sequence complementary to the 3′ terminal 18 nucleotides of $tRNA^{Lys3}$. Additional regions in $tRNA^{Lys3}$ and upstream of the PBS in viral RNA have also been proposed to be involved in the annealing (302).

Annealing of $tRNA^{Lys3}$ to viral RNA is facilitated by nucleocapsid proteins. Both the mature NCp7 and these sequences in the precursor Gag protein will facilitate annealing (69). HIV-1 nucleocapsid has two zinc fingers. The basic amino acids flanking the N-terminal zinc finger are required for $tRNA^{Lys3}$ annealing, but the two zinc fingers appear to have less effect (66, 120, 228, 318). The role of NCp7 in the annealing process appears to be two fold (227). One role is the denaturation of double-stranded RNA regions in viral RNA (such as the primer activation signal found upstream of the PBS (262), or a predicted 4 base pair helix predicted to be at the 5′ region of the PBS in one model (264)), which may be facilitated by the zinc fingers, and there is the nucleation step that brings $tRNA^{Lys3}$ and the PBS sequences together, which is probably facilitated primarily by the basic amino acids flanking the first zinc finger. The lack of effect on the rate of annealing by mutated zinc fingers is explained by the fact that while the mutant NCp7 is a weaker duplex destabilizer, it is a better duplex nucleating agent, and the two phenomenon may cancel each other out (227). NCp7 has also been predicted to play a role in blocking the interaction between the TΨC and D loops of $tRNA^{Lys3}$, thereby

facilitating a destabilization of tertiary structure which might promote annealing (228, 533).

Earlier studies have indicated that in protease-negative HIV-1, Gag is sufficient for annealing $tRNA^{Lys3}$ to the viral RNA (66, 154). However, the placement of $tRNA^{Lys3}$ on viral RNA is not optimal, since the incorporation of the first dNTP, dCTP, was reduced by 70% in an *in vitro* reverse transcription system consisting of total viral RNA as the source of the primer $tRNA^{Lys3}$/viral RNA template, exogenous HIV-1 RT, and a suboptimal concentration of dCTP (69). Transient exposure of the total viral RNA to mature NCp7 before reverse transcription restored incorporation of dCTP to levels obtained with total viral RNA isolated from protease-positive virions. This suggested that there might be two stages to annealing, one carried out by Gag, and one further adjustment carried out by NCp7. In this work, we provide further evidence for a weaker binding of $tRNA^{Lys3}$ annealed to viral RNA by Gag rather than by NCp7, by showing that $tRNA^{Lys3}$ annealed by Gag is more readily displaced from the PBS by reverse transcription initiated downstream of the PBS.

We also provide further evidence to support our previous reports (214, 215) on the ability of the anti-viral protein APOBEC3G (A3G) to inhibit $tRNA^{Lys3}$ annealing, and show that the ability of A3G to inhibit initiation of reverse transcription only applies to initiation from $tRNA^{Lys3}$ annealed by NCp7, and not from $tRNA^{Lys3}$ annealed by Gag. HIV-1 produced from cells expressing human APOBEC3G (A3G), also known as nonpermissive cells, have a severely reduced ability to productively infect other cells. A3G is incorporated into Vif-negative HIV-1 that are either protease-positive or protease-negative (65), and its incorporation depends upon the interaction of A3G with

nucleocapsid sequences in Gag (8, 65, 354, 491, 584). The presence of the HIV-1 protein Vif greatly inhibits this incorporation by interaction with A3G and targeting it for degradation via proteosomes (579). New infections by HIV-1 containing A3G are accompanied by a reduced production of viral DNA transcripts (200, 334, 367, 369). While it has been suggested that cytidine deamination by A3G of newly synthesized DNA results in this DNA's degradation (231, 322, 368, 585), other reports have indicated that the reduced viral DNA production is due to a direct inhibition of reverse transcription, occurring at both the initiation stage of reverse transcription (214, 215), and at the first and/or second DNA strand transfer stages (339, 379). The reduction in the initiation of reverse transcription is due to an inhibition of $tRNA^{Lys3}$ annealing to the viral RNA genome by A3G (214, 215). Although a recent publication reported that A3G does not inhibit the *in vitro* NCp7-facilitated $tRNA^{Lys3}$ annealing to viral RNA (267), we reported that A3G does inhibit the *in vitro* annealing of $tRNA^{Lys3}$ to viral RNA facilitated by mature nucleocapsid (NCp7), and demonstrated that the inhibition of $tRNA^{Lys3}$ annealing was dependent upon the interaction occurring between NCp7 and A3G (215). The inability of A3G to block initiation of reverse transcription from $tRNA^{Lys3}$ annealed by Gag is used to study the roles of Gag and NCp7 in promoting $tRNA^{Lys3}$ annealing in HIV-1.

3.4 Experimental Procedures

3.4.1 Plasmid construction

BH10 is a simian virus 40-based vector that contains full-length wild-type BH10 strain of HIV-1 proviral DNA, while BH10P- contains an inactive viral protease (D25G). Both plasmids were a gift from E. Cohen, University of Montreal. The construction of protease-positive, Vif-negative HIV-1 (BH10.Vif-), and protease-negative, Vif-negative HIV-1 (BH10P-Vif-) were generated by introducing into these plasmids a stop codon right after start codon (ATG) of the Vif reading frame at nt 5043, using a site-directed mutagenesis kit (Stratagene) and the following pairs of primers: 5′-AGATCATTAGGGATTTAGGAAAACAGATGGCAG-3′, and 5′-CTGCCATCTGTT TTCCTAAATCCCTAATGATCT-3′. The human pAPOBEC3G was constructed as previously described (65), and expresses wild type human APOBEC3G with a fused HA tag at the C terminus.

3.4.2 Protein production

Human A3G was obtained from Diagnostics, Inc. It was produced in baculovirus using the Baculo expression system, and was purified by $(NH_4)_2SO_4$ fractionation and immuno-affinity chromatography to >95% purity. HIV-1 RT, purified from bacteria as previously described (258), was a gift from Matthias Gotte (McGill University). Recombinant wild type NCp7 was a gift from R. Gorelick, and was prepared as previously described (62, 217), including purification by reverse-phase high-pressure liquid chromatography after denaturing the protein in 8 M guanidine hydrochloride.

GagΔp6 was a gift from A. Rein, and was prepared as previously described (69). GagΔp6 was used because it is easier to purify than full-length Gag, its assembly properties *in vitro* have been characterized (57), and it can efficiently facilitate annealing of $tRNA^{Lys3}$ to viral RNA (154).

3.4.3 Cells, transfections and virus purification

HEK-293T cells were grown in complete Dulbecco's modified Eagle's medium plus 10% fetal calf serum, 100 units of penicillin, and 100 μg of streptomycin/ml. For the production of viruses, HEK-293T cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, California) according to the manufacturer's instruction. Supernatant was collected 48 h post-transfection. Viruses were pelleted from culture medium by centrifugation in a Beckman Ti-45 rotor at 35,000 rpm for 1 h. The viral pellets were then purified by 15% sucrose onto a 65% sucrose cushion. The band of purified virus was removed and pelleted in 1X TNE (20 mM Tris, pH 7.8, 100 mM NaCl, 1 mM EDTA) in a Beckman Ti-45 rotor at 40,000 rpm for 1 h.

3.4.4 Viral RNA isolation and quantification

Total viral RNA was extracted from viral pellets using the guanidinium isothiocyanate procedure as previously described in (257) and was dissolved in 5 mM Tris buffer, pH 7.5. As previously described (69), hybridization to dot blots of total viral RNA was carried out with 5′-^{32}P-end-labeled DNA probes complementary to either the 3′-terminal 18 nucleotides of $tRNA^{Lys3}$ (5′-TGGCGCCCGAACAGGGAC-3′), or to the 5′ end of the HIV-1 genomic RNA, just upstream of the PBS (5′-CTGACGCTCTCGCA

CCC-3′). In addition to dot blot analysis for determining the amount of viral RNA used in each RT reaction mixture, the relative amounts of viral RNA in the reaction mixtures were also determined by measuring the ability of a 30-mer DNA (complementary to BH10 DNA nucleotides 801-830 (5′-TCTAATTCTCCCCCG CTTAATACTGACGCT-3′) annealed at room temperature to the viral RNA to prime synthesis of a 6-base deoxynucleotide triphosphate extension, using the same reaction conditions as for measuring $tRNA^{Lys3}$ priming, as previously described (215).

3.4.5 $tRNA^{Lys3}$ priming assay of reverse transcription

Total viral RNA isolated from virus produced in transfected 293T cells was used as the source of a primer tRNA annealed to viral RNA in an *in vitro* reverse transcription reaction as previously described (258). Briefly, total viral RNA was incubated at 37°C for 15 min in 20 μl of RT buffer (50 mM Tris-HCl (pH 7.5), 60 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol (DTT)) containing 50 ng purified HIV RT, 10 U of RNasin and various deoxynucleotide triphosphates (dNTPs). To measure the ability of annealed $tRNA^{Lys3}$ to be extended by six deoxyribonucleotides, the RT reaction mixture contained 200 μM dCTP, 200 μM dTTP, 5 μCi of [α-^{32}P] dGTP (0.16 μM), and 50 μM ddATP. To measure the ability of annealed $tRNA^{Lys3}$ to be extended by one deoxyribonucleotide, the RT reaction mixture contained only 0.16 μM α-^{32}P-dCTP. Reaction products were resolved using one-dimensional (1D) 6% polyacrylamide gel electrophoresis (PAGE) containing 7M urea (258).

3.4.6 *In vitro* annealing of $tRNA^{Lys3}$ to viral RNA.

Human placental $tRNA^{Lys3}$ was hybridized with synthetic HIV-1 viral genomic

RNA. The $tRNA^{Lys3}$ was purified from human placenta as previously described (275), using standard chromatography procedures (sequentially DEAE-Sephadex A-50, reverse-phase chromatography (RPC-5), and Porex C4) and, finally, two dimensional polyacrylamide gel electrophoresis (2D-PAGE). The synthetic genomic RNA (497 bases), comprising the complete R and U5 regions, the PBS, leader, and part of the *gag*-coding region, was synthesized as previously described (258) from the AccI-linearized plasmid pHIV-PBS (13) with the MEGAscript transcription system (Ambion Inc.). For heat annealing of $tRNA^{Lys3}$, 1 pmol of $tRNA^{Lys3}$ was combined with 1 pmol of synthetic HIV-1 RNA template in a solution containing 50 mM Tris-HCl (pH 7.5), 60 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 10 U of RNasin. The annealing reaction mixture was heated sequentially at 85°C for 5 min, at 50°C for 10 min, and then at 37°C for 15 min. $tRNA^{Lys3}$ annealing to viral RNA was facilitated by either the addition of NCp7 or GagΔp6 as previously described (69). 1 pmol of $tRNA^{Lys3}$ was annealed to 1 pmol of synthetic HIV-1 RNA template by incubating these reagents at 37°C for 90 min with 30 pmol of NCp7 or GagΔp6 in a 10 μl reaction mixture containing (50 mM Tris-HCl (pH 7.2), 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT) and 10 U of RNasin.

To study the effect of A3G on these annealing reactions, 1 pmol of either purified A3G or control bovine serum albumin (BSA), dissolved in 20 mM Tris-HCl (pH7.5), 0.1 M NaCl and 0.01% Sarcosyl, were added to the annealing reactions at the start. After the annealing, and in preparation for the $tRNA^{Lys3}$ priming reaction, the $tRNA^{Lys3}$/viral RNA complex was deproteinized through the addition of 1 μl proteinase K (5 mg/ml), and was incubated at 37°C for 30 min, followed by phenol/chloroform extraction to remove both

proteinase K and digested residual proteins, and ethanol precipitation of the viral RNA complex.

3.4.7 *In vitro* detection of the interaction between A3G and either NCp7or GagΔp6.

Equimolar amounts of either NCp7 or GagΔp6 were incubated with A3G for 2 h at 4°C in NET-gelatin buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% NP-40, and 0.25% gelatin). A3G was then immunoprecipitated from the mixture by incubation with anti-A3G rabbit antiserum (NIH AIDS Research and Reference Reagent Program) at 4°C overnight. Protein A-Sepharose CL-4B beads (Amersham PharmaciaBiotech) were added to the antibody-containing samples, and the samples were incubated for a further 1 h at 4°C to allow the beads to bind to the antibody-antigen complexes. The beads were washed three times with NET gelatin buffer containing 1% Triton X-100, and the bound proteins were heated in gel loading buffer and resolved by 1D sodium dodecyl sulfate (SDS)-PAGE. NCp7 or GagΔp6 were detected on Western blots probed with goat anti-NCp7 (a gift from Louis Henderson, AIDS Vaccine Program). Horseradish peroxidase-conjugated secondary antibodies used were anti-goat immunoglobulin (Rockland Immunochemicals). Protein bands were detected by enhanced chemiluminescence (Perkin-Elmer Life Science, Inc). Bands in Western blots were quantitated using ImageJ 1.35s public domain software (NIH).

3.4.8 Measurement of $tRNA^{Lys3}$ annealing to viral RNA.

Electrophoretic mobility shift assay— $tRNA^{Lys3}$ was 3′-end labeled with ^{32}pCp, as previously described (46). After deproteinization of the annealed complex with proteinase K, the free $tRNA^{Lys3}$ or DNA oligomer were resolved from primer complexed

with viral RNA using one dimension (1D) 6% native polyacrylamide gel electrophoresis (PAGE). *2. $tRNA^{Lys3}$ occupation of the PBS*— A 28 nt DNA oligomer (5′-CCCCGCT TAATACTGACGCTCTCGCACC-3′) was 5′-end labeled with ^{32}P using T4 kinase and was complementary to sequences in HIV-1 RNA genome 139 base downstream of the PBS. The complex was then incubated at 37°C for 30 min in 20 μl of RT buffer (50 mM Tris-HCl (pH 7.5), 60 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT) containing 50 ng HIV RT, 10 U of RNasin, 200 μM deoxynucleoside triphosphates, and reaction products, either full length (365 nt) or truncated (167 nt, due to occupation of the PBS by $tRNA^{Lys3}$), were resolved using 1D 6% PAGE with gels containing 7M Urea.

3.5 Results

3.5.1 A3G inhibits initiation of reverse transcription in protease-positive virions, but not in protease-negative virions.

In this work, Vif-negative HIV-1 were used to allow for the incorporation of A3G into the viruses. 293T cells were cotransfected with a plasmid coding for either protease-positive, Vif-negative HIV-1 (BH10Vif-) or protease-negative, Vif-negative HIV-1 (BH10P-Vif-), and a plasmid coding for, or not coding for, A3G. We have previously reported that the absence of Vif has no effect upon $tRNA^{Lys3}$ incorporation or initiation of reverse transcription in virions produced from permissive 293T cells (214). Total viral RNA was isolated from each of these four types of viruses produced (protease-positive or protease-negative, with or without A3G), and the content of viral genomic RNA and $tRNA^{Lys3}$ in the total viral RNA was determined by hybridizing dot blots of the RNA with 5′-^{32}P-end-labeled DNA probes complementary to either the 3′ terminal 18 nucleotides of $tRNA^{Lys3}$, or to the 5′ end of the HIV-1 genomic RNA,

upstream of the PBS. Figure 3.1A shows that all 4 types of virions have equal incorporation of $tRNA^{Lys3}$ into virions (i.e., equal $tRNA^{Lys3}$/viral RNA ratios). Thus $tRNA^{Lys3}$ incorporation is not affected either by the inability to process viral proteins or by the presence of A3G in the cell and virus.

Initiation of reverse transcription was measured in an *in vitro* reverse transcription assay that extends the $tRNA^{Lys3}$ by 6 nt, using total viral RNA was used as the source of primer $tRNA^{Lys3}$ annealed to viral RNA (see the cartoon in Figure 3.1B). Reaction products were resolved using 6% 1D PAGE. Figure 3.1C, left, shows the 3.1D PAGE pattern of the 6 nt-extended $tRNA^{Lys3}$ produced from the RNA isolated from each viral type, the results of which are graphed in Figure 3.1C, right, normalized to those values produced using BH10Vif- viral RNA. As previously reported (69), 6 nt extension of $tRNA^{Lys3}$ in protease-negative viruses is reduced approximately 25% compared to protease-positive viruses. Also, in protease-positive virions the presence of A3G reduces relative initiation of reverse transcription to 40% of that obtained in the absence of A3G. However, A3G has little effect on initiation in protease-negative viruses.

As we have previously reported (69), the +6 nt extension assay contains an excess of dNTPs which hides more subtle differences in the ability of $tRNA^{Lys3}$ to initiate reverse transcription when annealing of the $tRNA^{Lys3}$ has occurred in either a protease-negative or protease-positive envirionment. Such differences can be detected when the first dNTP incorporated, dCTP, is present at sub-optimal quantities in the reverse transcription assay. This is shown in Figure 1D, in which the $tRNA^{Lys3}$ priming assay using total viral RNA as the source of primer/ template occurs in the presence of only one dNTP, 0.16 μM α-^{32}P-dCTP. The ability of protease-negative viruses to

incorporate dCTP is reduced to less than 20% of that found for viral RNA from protease-positive viruses, independently of the presence of A3G. We also show that the presence of A3G in protease-positive viruses reduces the +1 extension to 26% of that found for protease-positive virions not containing A3G, an even larger reduction than we report for the 6 nt extension assay (Fig.1). As also seen for +6 extension (Fig.1C), A3G shows no effect upon +1 nt extension in protease-negative virions.

3.5.2 The effect of lack of viral protein processing and/or A3G upon the occupancy of the primer binding site by $tRNA^{Lys3}$.

Diffferences in the ability of RNA from these different viruses to extend $tRNA^{Lys3}$ by 6 nt may reflect similar differences in $tRNA^{Lys3}$ annealing that occur *in vivo*. To further investigate this, we have used another assay that more directly measures $tRNA^{Lys3}$ annealing by determining the fraction of primer binding sites (PBSs) occupied by $tRNA^{Lys3}$. A cartoon of the experimental strategy is shown in Figure 3.2A. A 5′-^{32}P-end-labeled DNA primer (28 nt) is annealed to a sequence in total viral RNA 139 nt downstream of the PBS, and is then extended with HIV-1 RT. Full length extension will give a labeled 365 nucleotide product, but $tRNA^{Lys3}$ annealed at the PBS will block extension, resulting in a 167 nucleotide product. Resolution of these products by 1D PAGE is shown in Figure 3.2B, with the percentage of the PBSs that are occupied (167 nucleotide product/167 + 365 nucleotide products) listed below each lane. The first two lanes show that the PBS occupancy by $tRNA^{Lys3}$ in BH10Vif- (i.e., protease-positive) is reduced from 74% occupancy to 31% occupancy when A3G in present, i.e., A3G reduces annealing by 58%. This number is very similar to the 60% reduction in initiation measured using the $tRNA^{Lys3}$ 6 nt extension assay (Figure 3.1C), demonstrating that

changes in $tRNA^{Lys3}$ initiation of reverse transcription reflect changes in the amount of $tRNA^{Lys3}$ annealed to the PBS.

The value of 74% occupancy of the PBS achieved in the absence of A3G may be considered a minimum value since we do not know if the block to elongation by $tRNA^{Lys3}$ annealed to the PBS is 100% efficient. On the other hand, since we have previously shown in an *in vitro* $tRNA^{Lys3}$ annealing system that PBS occupancy is only 1% when $tRNA^{Lys3}$ is absent in a system containing both synthetic viral RNA and NCp7 (215), it is not likely that an unoccupied PBS is in a configuration that can partially block RT elongation. The two lighter bands that move slightly slower than 167 nt band, are also seen in the *in vitro* system with no $tRNA^{Lys3}$ present (215), suggesting these bands are due to natural pause sites of DNA synthesis that occur independently of PBS occupancy.

The +6 extension data seen in Fig.1C suggests that the amount of $tRNA^{Lys3}$ annealed to viral RNA isolated from protease-negative viruses is at least 75% that of the value found in protease-positive virions, and that the much lower +1 extension (20% that of the value found in protease-positive virions (Fig.1D)) may therefore be due to a non-optimal placement of the annealed $tRNA^{Lys3}$ on the viral RNA that lessens its ability to incorporate the first nucleotide. This proposed weaker binding of $tRNA^{Lys3}$ to viral RNA in protease-negative viruses is also suggested by measurement of PBS occupancy in protease-negative viruses. For while +6 nt extension is only reduced 25% in protease-negative virions, PBS occupancy is reduced 73%, and independently of the presence or absence of A3G.

3.5.3 The inability of A3G to inhibit reverse transcription using RNA from protease-negative viruses is supported by the inability to inhibit Gag-facilitated $tRNA^{Lys3}$ annealing *in vitro*.

The $tRNA^{Lys3}$ +6 extension results in Fig.1 suggest that A3G does not affect initiation of reverse transcription from $tRNA^{Lys3}$ annealed to viral RNA by Gag. We have previously reported that the NCp7-facilitated *in vitro* annealing of $tRNA^{Lys3}$ to viral RNA is inhibited by A3G, as measured either indirectly by +6 nt extension of $tRNA^{Lys3}$ (70-80% inhibition) or by PBS occupancy (60% inhibition) (215). We therefore examined next whether A3G can inhibit +6 nt extension of $tRNA^{Lys3}$ annealed by GagΔp6 *in vitro* in the presence of either BSA or A3G. In order to see differences in the ability between NCp7 and GagΔp6 to initiate reverse transcription, annealing of $tRNA^{Lys3}$ by either protein should not saturate the PBS with $tRNA^{Lys3}$. We have previously shown that initiation of reverse transcription (+6 nt extension) increases linearly when one picomole $tRNA^{Lys3}$ is annealed to one picomole synthetic HIV-1 RNA using from 30 to 60 picomoles NCp7 (215). We therefore used 30 picomoles in the *in vitro* annealing system, which was also determined in that paper to result in 47% occupancy of the PBS. In that work, we also observed that maximum inhibition of the initiation of reverse transcription occurs with an A3G:NCp7 ratio = 1:30, and so used one picomole A3G in the annealing reaction for our inhibition studies.

The 6 nt RT extension products of $tRNA^{Lys3}$ are resolved using 6% 1D PAGE containing 7M urea (Figure 3.3A). Normalizing to +6 nt extension from $tRNA^{Lys3}$ annealed by NCp7 in the absence of A3G, we see that +6 nt extension from $tRNA^{Lys3}$ annealed by GagΔp6 is approximately 70% of that achieved by NCp7-facilitated annealing. The presence of A3G in the NCp7 annealing mixture reduces +6 nt extension

to approximately 16%, but has no effect upon initiation when added to a GagΔp6 annealing mixture, thereby supporting our *in vivo* findings that initiation of reverse transcription from $tRNA^{Lys3}$ annealed by Gag is insensitive to A3G (Figure 3.1C).

Figure 3.3B repeats the experiment in Figure 3.3A, but shows the results of +1 nt extension. A3G shows a similar inhibition of +1 nt extension of $tRNA^{Lys3}$ annealed by NCp7, but as demonstrated in Figure 3.1 for RNA isolated from Pr- viruses, +1 nt extension of $tRNA^{Lys3}$ annealed by GagΔp6 is highly reduced, with or without the presence of A3G. The +6 extension data seen in Figure 3.1 or in Figure 3.3A indicate that this reduced +1 nt extension for Gag-annealed $tRNA^{Lys3}$ is due less to the amount of $tRNA^{Lys3}$ annealed, and more to its weakened placement on the viral RNA (Figure 3.2B). This is further indicated by the data shown in Figure 3.3C, where, using the electrophoretic band shift method to resolve free from annealed $tRNA^{Lys3}$, we directly measure the amount of labeled $tRNA^{Lys3}$ annealed by either NCp7 or GagΔp6, in the presence or absence of A3G. In our annealing conditions, NCp7 in the absence of A3G anneals approximately 37% of $tRNA^{Lys3}$ to the viral RNA, and there is a 55% reduction when A3G is present. GagΔp6 anneals in approximately 85% of the $tRNA^{Lys3}$ annealed by NCp7, with little change occurring when A3G is also present during annealing.

We have previously reported that the ability of A3G to inhibit initiation of reverse transcription from $tRNA^{Lys3}$ annealed by NCp7 *in vitro* depends upon the interaction of A3G with NCp7 (215). We therefore investigated whether the failure of A3G to inhibit GagΔp6–facilitated $tRNA^{Lys3}$ annealing *in vitro* resulted from the inability of A3G to interact with Gag. As described previously for measuring the NCp7/A3G interaction *in vitro* (215), equimolar amounts of either NCp7 or GagΔp6

were incubated with A3G, and their ability to be coimmunoprecipitated with anti-A3G (NIH AIDS Research and Reference Reagent Program) was detected on Western blots probed with goat anti-NCp7 (gift from Louis Henderson, AIDS Vaccine Program). The results, shown in Figure 3.3D, demonstrate that both NCp7 and GagΔp6 interact efficiently with A3G.

3.5.4 A3G can reduce initiation of reverse transcription using $tRNA^{Lys3}$ annealed by GagpΔ6 if the annealed complex is exposed to NCp7.

Figures 3.1 and 3.3 show that $tRNA^{Lys3}$ annealing by Gag is insensitive to A3G, and Figure 3.1 also shows that initiation of reverse transcription from RNA isolated from protease-negative viruses is still 70-80% that found for RNA from protease-positive viruses. If we assume that $tRNA^{Lys3}$ annealing is exposed to both Gag and NCp7 in a protease-positive virus, then how is A3G able to reduce initiation of reverse transcription by 60% in the protease-positive virus, and not retain the A3G-resistant initiation of reverse transcription achieved in protease-negative viruses? To answer this, we examined the effect that exposure of a Gag-annealed $tRNA^{Lys3}$/viral RNA complex to NCp7 might have upon the resistance of initiation of reverse transcription to A3G. The results are shown in Figure 3.4. $tRNA^{Lys3}$ was first annealed *in vitro* to viral RNA using either NCp7 (left side) or GagΔp6 (right side), deproteinized, transiently exposed to BSA, NCp7, or GagΔp6, again deproteinized, and then analyzed for +6 nt extension. We have previously shown that once $tRNA^{Lys3}$ is annealed by NCp7 *in vitro*, A3G is unable to disrupt initiation of reverse transcription, i.e., A3G inhibition occurs during annealing, not afterwards (215). Similar results are shown in the left region of Fig. 4. However, it can be seen that while +6 nt extension from $tRNA^{Lys3}$ annealed by GagΔp6 is also

resistant to A3G, transient exposure of this complex to NCp7 in the presence of A3G results in a significant decrease in initiation. These results support the previously published observation that *in vitro* exposure of viral RNA from protease-negative HIV-1 to NCp7 rescues +1 nt extension of $tRNA^{Lys3}$ (69), and suggests that NCp7 alters the conformation of $tRNA^{Lys3}$ annealed to viral RNA by Gag.

3.6 Discussion

While the initiation of reverse transcription (+6 nt extension) from a $tRNA^{Lys3}$ annealed by Gag (i.e, from viral RNA isolated from protease-negative virions) is 20-30% lower than in protease-positive virions (Figure 3.1C), the ability of this tRNA to incorporate the first deoxynucleotide, dCTP, present at sub-optimal concentrations in the reaction, is much lower (>80% reduction) than for $tRNA^{Lys3}$ annealed in protease-positive viruses (Figure 3.1D), suggesting a sub-optimal annealing in a protease-negative environment. The postulated weaker interaction of $tRNA^{Lys3}$ annealed to viral RNA by Gag is further supported by the PBS occupancy data shown in Figure 3.2, which shows a 73% reduction in occupancy, even though +6 base extension is approximately 70-80% that found for protease-positive viruses. This most likely reflects the weaker annealing of $tRNA^{Lys3}$ in protease-negative virions that will allow DNA extension from the downstream DNA primer to displace $tRNA^{Lys3}$ annealed to the PBS. The conditions used in the 6 nt extension assay, including the high concentrations of dNTPs, appear to be able to overcome the differences in annealing configurations in protease-positive and protease-negative virions, allowing for more similar initiation of reverse transcription.

We also studied the effect of A3G upon both initiation of reverse transcription and $tRNA^{Lys3}$ annealing in protease-positive and protease-negative viruses, using naturally permissive 293T cells expressing exogenous A3G. We have previously reported that $tRNA^{Lys3}$ initiation of reverse transcription is inhibited in the naturally non-permissive H9 and MT2 cell lines, compared with that occurring in the permissive MT4 cell line. Levels of inhibition of initiation similar to that found in H9 cells can be observed in 293T cells expressing exogenous A3G, but only at a 10 fold greater expression of exogenous A3G (214). This suggests that the inhibition of initiation in non-permissive cells requires the presence of additional factors besides A3G that may not be present in proper amounts or locations in permissive cells. However, because the degrees of inhibition of $tRNA^{Lys3}$ annealing achieved are similar in these two cell types, we propose that the mechanism by which A3G inhibits reverse transcription initiation are likely to be the same in both cell types. A3G does not affect the incorporation of $tRNA^{Lys3}$ into BH10Vif- (Figure 3.1A), but it does reduce +6 nt extension of $tRNA^{Lys3}$ by 60% (Figure 3.1C). This reduction correlates with a similar reduction in PBS occupancy shown in the left 2 lanes in Figure 3.2B, indicating that A3G inhibits $tRNA^{Lys3}$ annealing. Thus, while the conditions in the +6 nt extension assay, including the high concentrations of dNTPs, are able to overcome the differences in annealing configurations between protease-positive and protease-negative virions, allowing for more similar initiation of reverse transcription, these same conditions cannot overcome the inhibitory effect of A3G on initiation of reverse transcription in protease-positive virions. Also, as mentioned above, the PBS occupancy test cannot be applied to protease-negative virions containing A3G because the weaker placement of $tRNA^{Lys3}$ on

the PBS does not not appear to be able to block extension of the downstream DNA primer.

Our results comparing protease-positive and protease-negative viruses might suggest that $tRNA^{Lys3}$ annealing *in vivo* could involve two consecutive steps. The first one catalyzed by Gag would result in less than optimal annealing for efficient initiation of reverse transcription. The role of nucleocapsid sequences in Gag might be to bring the largely single-stranded PBS sequences and the $tRNA^{Lys3}$ together through electrostatic interactions with basic amino acids flanking the first zinc finger. The main role of NCp7 might be to optimize $tRNA^{Lys3}$ placement on the viral RNA by destabilizing double-stranded regions in the viral RNA which can also interact with $tRNA^{Lys3}$, such as the primer activation signal, and to destabilize the $tRNA^{Lys3}$ tertiary structure through blocking the D and TΨC loop interaction.

However, our results with A3G do not fit with this simple consecutive two-step annealing process. The inhibition of $tRNA^{Lys3}$ annealing to viral RNA by A3G requires an interaction between NCp7 and A3G (215), implying that A3G may be blocking the annealing function of NCp7. This is also supported by the observation that if A3G is added only after $tRNA^{Lys3}$ is annealed in vitro using NCp7, annealing is not disrupted (215) of It is not clear why A3G is unable to inhibit Gag-facilitated $tRNA^{Lys3}$ annealing, but this fact does help us to dissect the annealing process. For if the annealing of $tRNA^{Lys3}$ in protease-positive viruses is a consecutive two step process, we would expect A3G to reduce $tRNA^{Lys3}$ priming to that level first obtained by Gag alone, i.e., A3G should only reduce +6 nt extension of $tRNA^{Lys3}$ the 20-30% it achieves in Pr- virions. But the results in Figures 3.1C show that in protease-positive viruses, A3G causes a 60%

inhibition of +6 nt extension, suggesting that the presence of NCp7 alters the annealing achieved by Gag alone. While it is possible that $tRNA^{Lys3}$ annealing in Pr+ viruses occurs only after Gag is processed to NCp7, it is also possible that Gag annealing occurs first, and is then altered by later production of NCp7. This latter interpretation is supported by the data in Figure 3.4, which shows that the initiation of reverse transcription from an in annealed complex formed by Gag *in vitro* can become sensitive to A3G upon a subsequent transient exposure to NCp7, and also reflects a previous report showing that the ability of viral RNA isolated from Pr- virions to incorporate the first dCTP during initiation of reverse transcription can be brought to wild type levels by transient exposure to NCp7 (69).

If, as observed *in vitro* (215), A3G blocks $tRNA^{Lys3}$ annealing through its interaction with NCp7, how could the relatively few A3G molecules in the virus (estimated to be 7 +/-4 in HIV-1 produced from H9 cells (574)) non-enzymatically affect $tRNA^{Lys3}$ annealing through its interaction with the much greater number of NCp7 molecules, which have been estimated to be present at 1400 (595) to 5000 (42) molecules/virion? There are a number of possibilities. First, the A3G/NCp7 ratio determined for mature virions may not represent the A3G/NCp7 ratio at the stage of viral assembly where $tRNA^{Lys3}$ annealing occurs, and evidence has been reported supporting a multistage process for viral assembly (222, 346). Second, A3G may concentrate at or near the site where $tRNA^{Lys3}$ annealing occurs. For example, Gag has been shown to have a preference for binding to stem-loop structures in the 5′ region of the viral RNA that include the PBS stem loop (88). Also, annealing of $tRNA^{Lys3}$ to viral RNA *in vivo* may require more proteins than just NCp7, some of which might bind to

A3G and bring it close to the site of annealing. Examples of non-nucleocapsid proteins known to be able to regulate $tRNA^{Lys3}$ annealing include reverse transcriptase (71) and Tat (285), and roles for cellular RNA helicases and lysyl-tRNA synthetase (LysRS) in this process are currently being explored.

Thus, $tRNA^{Lys3}$ annealing *in vivo* may be more complex than simply exposing $tRNA^{Lys3}$ and viral RNA to Gag or NCp7, as is done *in vitro*. For example, during the incorporation of $tRNA^{Lys3}$ into the virion, Gag is only indirectly associated with $tRNA^{Lys3}$ through the $tRNA^{Lys3}$-binding protein, lysyl-tRNA synthetase (70). The packaging of $tRNA^{Lys3}$ requires the presence of RT sequences in GagPol (134, 296), and therefore a transfer of $tRNA^{Lys3}$ from the LysRS bound to Gag to the RT sequences in GagPol may be required (480). Whether this results in a further transfer of $tRNA^{Lys3}$ from RT to viral RNA is unknown, but a direct role of RT in $tRNA^{Lys3}$ annealing *in vivo* has been reported (71). It is therefore very important to support *in vitro* analyses of the effect of A3G upon Gag-or NCp7-facilitated $tRNA^{Lys3}$ annealing with data obtained for *in vivo* annealing. The reasons why we believe that total viral RNA used in these experiments is a source of $tRNA^{Lys3}$ annealed *in vivo* to viral RNA have been given elsewhere (214).

Thus, a recent publication reported that A3G does not inhibit NCp7-facilitated $tRNA^{Lys3}$ annealing *in vitro* to viral RNA sequences (267). Their conditions appear to promote more annealing of labeled $tRNA^{Lys3}$ than we obtain. It is therefore possible that their failure to show the A3G inhibition of annealing that we have observed using $tRNA^{Lys3}$ annealed both *in vivo* and *in vitro* may be because the annealing conditions

they use might drive the annealing reaction far enough to the right so as to make it less susceptible to regulation by exogenous molecules such as A3G.

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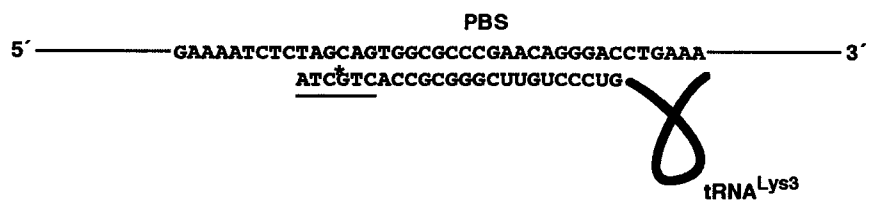
Figure 3.1 $tRNA^{Lys3}$ incorporation and initiation of reverse transcription in protease-positive and protease-negative HIV-1, lacking or containing A3G.

Four types of virions were produced from 293T cells cotransfected with BH10Vif- or BH10P-Vif- and a plasmid coding or not coding for A3G. Total viral RNA was isolated. **A.** Relative incorporation of viral genomic RNA and $tRNA^{Lys3}$ into virions, as determined by dot blot hybridization of total viral RNA with a probe specific for either viral RNA or $tRNA^{Lys3}$. The values were normalized to that obtained for BH10Vif- produced in the absence of A3G. **B-D.** Relative initiation of reverse transcription. **B.** A cartoon of the $tRNA^{Lys3}$/genomic RNA complex, with the 6 nt extension by reverse transcriptase underlined. **C.** 6 nt extension of $tRNA^{Lys3}$. Total viral RNA was used as the source of primer/template in an *in vitro* reverse transcription system, as described in text. In the reaction mix, dGTP is labeled, and dATP is replaced with ddATP. Shown are the resolution by 1D PAGE of the 6 nt extension products of $tRNA^{Lys3}$ (left), and the quantitation of the amount of 6 nt extension per equal amount of genomic RNA, relative to that obtained for BH10Vif- (right). **D.** One nt (dCTP) extension of $tRNA^{Lys3}$. Total viral RNA was used as the source of primer/template in an *in vitro* reverse transcription system, as described in text, whose only dNTP is labeled dCTP, present at sub-optimal concentrations (0.16 μM). Shown are the resolution by 1D PAGE of the 1 nt extension products of $tRNA^{Lys3}$ (left), and the quantitation of the amount of 1 nt extension per equal amount of genomic RNA, relative to that obtained for BH10Vif- (right).

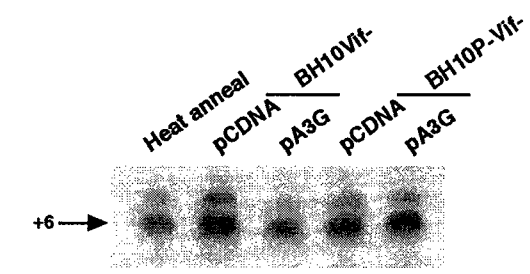
A

	BH10Vif-		BH10P-Vif-	
	pcDNA	pA3G	pcDNA	pA3G
Genomic RNA	1.00	1.04	0.93	0.96
tRNA ^{Lys3}	1.00	0.97	0.95	1.02

B



C



D

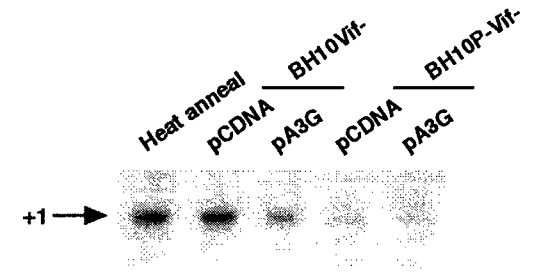
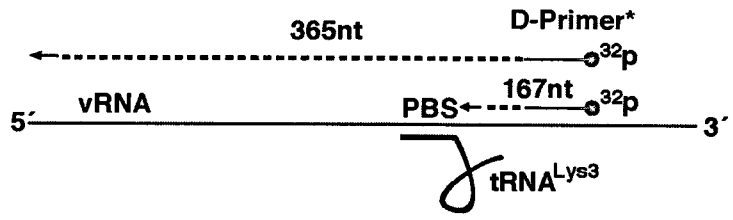


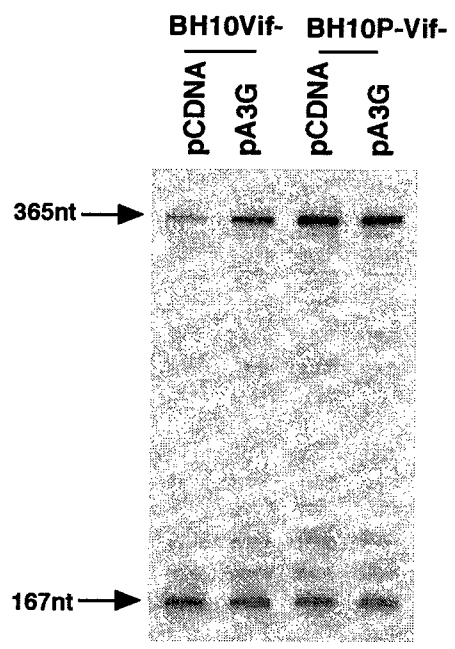
Figure 3.2 Measurements of the occupancy of the primer binding site by $tRNA^{Lys3}$ in protease-positive and protease-negative HIV-1, lacking or containing A3G.

Total viral RNA samples isolated from the four virus types listed in Figure 3.1 were used to determine primer binding site occupancy by $tRNA^{Lys3}$. **A.** Cartoon showing the strategy used to measure the percentage of the PBSs occupied with $tRNA^{Lys3}$. A 5′-^{32}P end-labeled DNA primer (28 nt) was annealed 139 bases downstream of the PBS in viral RNA, and extended with reverse transcription. The full-length extension product is 365 nt, while a truncated product, resulting from blocked extension by the presence of $tRNA^{Lys3}$ on the PBS, is 167 nt. **B.** Separation of the full-length and truncated extension products by 1D PAGE. The ratio of truncated product:truncated and full-length product, X 100 = the percentage of PBSs occupied by $tRNA^{Lys3}$, which is listed at the bottom of each lane.

A



B

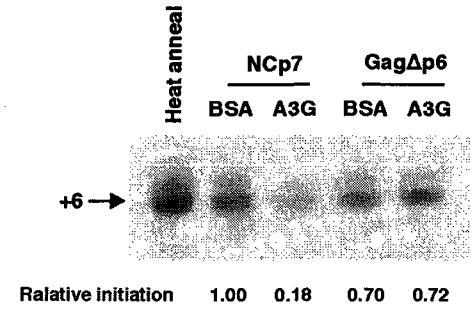


PBS Occupancy (%) 74 31 19 22

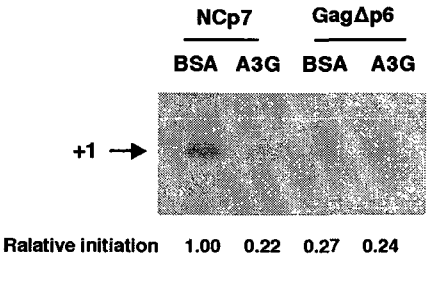
Figure 3.3 The effect of A3G on the GagΔp6- or NCp7-facilitated annealing of $tRNA^{Lys3}$ to viral RNA *in vitro*.

One picomole synthetic viral genomic RNA and one picomole purified human placental $tRNA^{Lys3}$ were incubated with 30 picomoles of either GagΔp6 or NCp7, in the presence or absence (replaced with BSA) of A3G. **A,B.** The resulting $tRNA^{Lys3}$/viral RNA complex was then used as a source of primer $tRNA^{Lys3}$/template viral genomic RNA in an *in vitro* reverse transcription system containing HIV-1 RT and deoxynucleotides, to produce either a 6 nt (A) or 1 nt (B) extension of $tRNA^{Lys3}$, as described in the legend to Figure 3.1. Shown are 1D PAGE patterns of $tRNA^{Lys3}$ extended by either 6 (A) or 1 (B) nts, with quantitation of the results, relative to that of NCp7-facilitated annealing in the absence of A3G, shown below each lane. **C.** The same annealing conditions as used for experiments in panels A and B were used with labeled $tRNA^{Lys3}$, and annealed vs free $tRNA^{Lys3}$ were resolved by electrophoresis. The fraction of total $tRNA^{Lys3}$ that was annealed is shown below each lane. **D**. The interaction of 30 picomoles of GagΔp6 or NCp7 with A3G. GagΔp6 or NCp7 was incubated with 1 picomole A3G, and the ability of GagΔp6 or NCp7 to be coimmunoprecipitated with A3G, using anti-A3G, was detected by Western blots probed with anti-NCp7, as described in the text. The top two panels are Western blots of the reaction prior to immunoprecipitation, probed with anti-A3G or anti-NCp7, respectively. The lower panel is a Western blot of the anti-A3G immunoprecipitate from each reaction, probed with anti-NCp7.

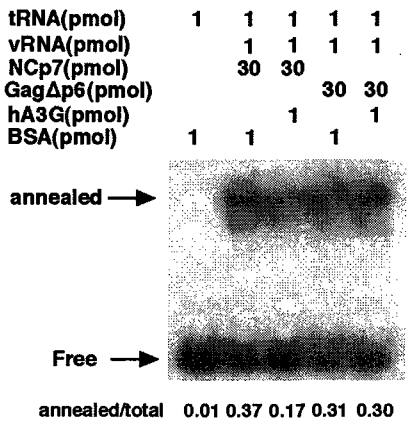
A


Heat anneal
NCp7
GagΔp6
BSA A3G BSA A3G
+6
Ralative initiation 1.00 0.18 0.70 0.72

B


NCp7
GagΔp6
BSA A3G BSA A3G
+1
Ralative initiation 1.00 0.22 0.27 0.24

C


tRNA(pmol) 1 1 1 1 1
vRNA(pmol) 1 1 1 1
NCp7(pmol) 30 30
GagΔp6(pmol) 30 30
hA3G(pmol) 1 1
BSA(pmol) 1 1 1
annealed
Free
annealed/total 0.01 0.37 0.17 0.31 0.30

D

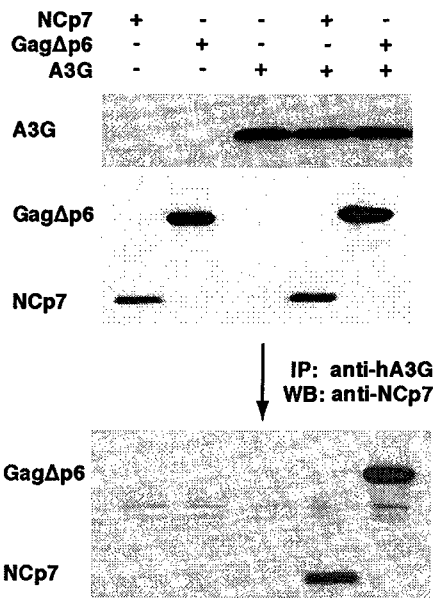
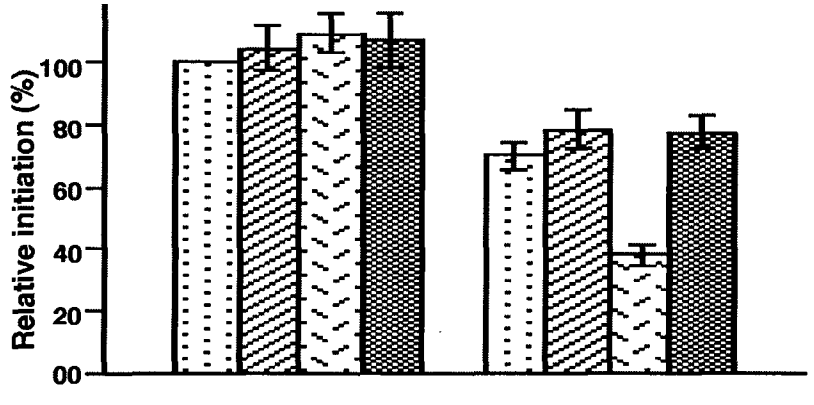
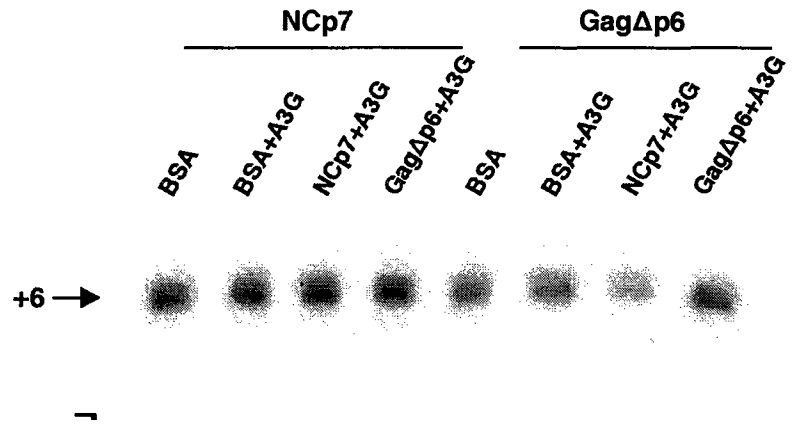

NCp7 + - - + -
GagΔp6 - + - - +
A3G - - + + +
A3G
GagΔp6
NCp7
IP: anti-hA3G
WB: anti-NCp7
GagΔp6
NCp7

Figure 3.4 The effect of GagΔp6 or NCp7 upon the inhibition by A3G of $tRNA^{Lys3}$ priming from a GagΔp6-facilitated annealed complex.

One picomole synthetic viral genomic RNA and one picomole purified human placental $tRNA^{Lys3}$ were incubated with 30 picomoles of either GagΔp6 or NCp7, as described in the text. The resulting $tRNA^{Lys3}$/viral RNA complex was then deproteinized, and used as a source of primer $tRNA^{Lys3}$/template viral genomic RNA in an *in vitro* reverse transcription system containing HIV-1 RT and deoxynucleotides so as to measure +6 nt priming. Reverse transcription took place in the presence of one picomole BSA alone, or in the presence of one picomole A3G and either BSA, NCp7, or GagΔp6. The upper panel shows 1D PAGE patterns of $tRNA^{Lys3}$ extended by 6 nts, and the lower panel shows quantitation of the results, relative to that of the +6 nt extension from an NCp7-annealed complex in the presence of BSA alone.


Relative initiation (%)
100
80
60
40
20
00


NCp7
GagΔp6
BSA
BSA+A3G
NCp7+A3G
GagΔp6+A3G
BSA
BSA+A3G
NCp7+A3G
GagΔp6+A3G
+6

3.7 Addendum

Interaction of APOBEC3G with cellular LysRS

(Here represented recent unpublished data (2008))

A3G has been shown to be able to inhibit $tRNA^{Lys3}$ annealing to the HIV-1 genome both *in vivo* and *in vitro*, independently of enzyme activity, and this inhibition requires interaction of A3G with NCp7. Because the number of A3G molecules in each virion are low (7 +/- 4) compared to the number of NCp7 molecules in a mature virion (approx. 1800) it is not clear how such a small number of A3G molecules can accomplish this job independently of enzyme activity. We have hypothesized that A3G may bind to molecules involved in the $tRNA^{Lys}$ packaging/annealing complex, which could bring A3G closer to the region of annealing. In Figure 3.5, we present data showing that A3G interacts with one such molecule, LysRS. In panel A, lysates of 293T cells and 293T cells transfected with a plasmid coding for HA-tagged A3G were examined for the ability of LysRS to interact with A3G. The left side of the panels in A represents cellular expression of A3G, GAPDH, and β–actin, i.e., they represent blots of cell lysate probed with antibodies to these different proteins. LysRS was immunoprecipitated from cell lysate with anti-LysRS, and Western blots of the immunoprecipitate probed with either anti-A3G (right, top panel) or anti-HA (right, second panel) show that A3G interacts with LysRS. The left side of the bottom two panels shows that this interaction is specific, i.e., the anti-LysRS immunoprecipitate does not contain two common cellular proteins, GAPDH or β–actin. Panel B shows that this interaction is RNA-independent, i.e., treatment of the anti-LysRS immunoprecipitate with RNaseA and T1 RNase does not disrupt the LysRS/A3G interaction.

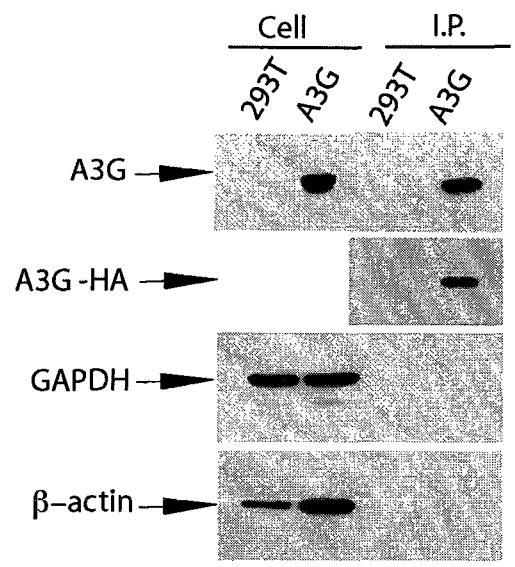
Future work in this area will be to determine if in fact LysRS binds to viral genomic RNA, and if it shows a binding preference to stem loops in the area of the primer binding site.

Figure 3.5 Interaction of endogenous cellular LysRS with APOBEC3G.

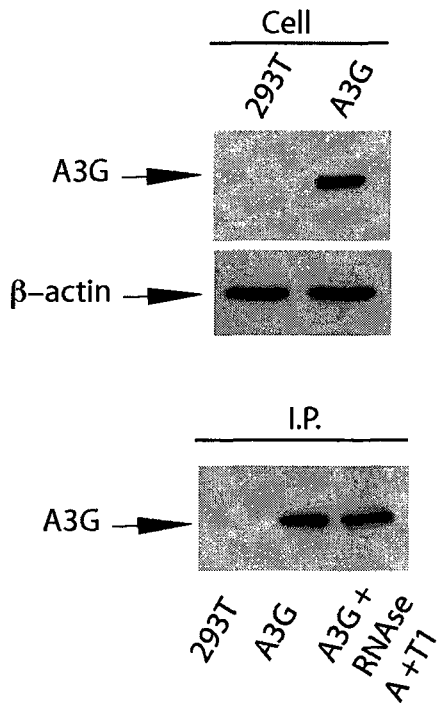
293T cells were transfected with a plasmid coding for wild-type HA-tagged full length APOBEC3G. **A.** Cellular expression of APOBEC3G, and its interaction with LysRS. Western blots of cellular lysates were probed with anti-APOBEC3G (left first panel). Interaction of APOBEC3G with endogenous LysRS was determined by immunoprecipitation of cell lysates with anti-LysRS, followed by probing western blots of the anti-LysRS immunoprecipitates with anti-APOBEC3G (right first panel) or anti-HA (right second panel). The specificity of the APOBEC3G and LysRS interaction was also examined. Western blots of the lysates of cells expressing or not expressing A3G were probed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (left third panel), or anti-β-actin (left fourth panel) to measure cell expression. Western blots of anti-LysRS immunoprecipitates from these cell lysates were probed with anti-GAPDH (right third panel) or anti-β-actin (right fourth panel).

(B) RNA-independence of the APOBEC3G/LysRS interaction. Lysates of cells expressing APOBEC3G and β-actin (western blots shown in the upper two panels) were immunoprecipitated with anti-LysRS. The immunoprecipitate was, or was not, treated with RNase A and T1 RNase, and the bottom panel shows a western blot of untreated or treated immunoprecipitate probed with anti-APOBEC3G.

A. Immunoprecipitation of A3G with anti-LysRS



B. RNAse A and T1 treatment of the immunoprecipitates of A3G with anti-LysRS, detected with anti-HA



CHAPTER IV

GENERAL DISCUSSION

Conversion of the HIV-1 RNA genome to DNA by reverse transcriptase is a key step in the HIV-1 viral life cycle. The initiation of reverse transcription is primed by a cellular tRNA, $tRNA^{Lys3}$, that is annealed to the viral RNA genome. This tRNA, along with the other major $tRNA^{Lys}$ isoacceptors, $tRNA^{Lys1}$ and $tRNA^{Lys2}$ (all together referred to as $tRNA^{Lys}$), are selectively packaged into the virus during HIV-1 assembly, and this concentration of $tRNA^{Lys3}$ in the virion has been shown to be important for the extent of both annealing in, and infectivity of, a viral population (174). The selective incorporation of $tRNA^{Lys}$ appears to result from the ability of Gag to specifically bind to LysRS, and incorporate it into the virion (70). However, $tRNA^{Lys}$ will not be packaged unless GagPol is also present (296, 358). As shown in Figure 2.5 A in Chapter 2, we have proposed a model of how a $tRNA^{Lys}$ packaging/annealing complex is formed during early viral assembly, in which a Gag/GagPol/viral RNA complex interacts with a $tRNA^{Lys}$ /LysRS complex, with Gag interacting with both GagPol and LysRS, and GagPol also interacting with $tRNA^{Lys}$ and keeping it in the complex. As written in the discussion section of Chapter 2, we believe this $tRNA^{Lys}$ packaging/annealing complex represents an early assembly intermediate in virus formation, which contains all the GagPol and viral RNA present in the immature virion, but to which more Gag molecules will be added prior to budding. In panel B in that figure, we propose relationships between the various protein and RNA components comprising this complex, which is discussed in detail in the discussion section of Chapter 2. The models in this figure are based on biochemical data, since the actual structures of Gag and GagPol have not been determined.

Two processes that are likely to occur within this complex are the transfer of $tRNA^{Lys3}$ from LysRS to the RT thumb sequences in GagPol, and the further transfer of $tRNA^{Lys3}$ from GagPol to the PBS sequence in viral RNA, where annealing facilitated by Gag and NCp7 takes place. The investigation in Chapter 2 was an attempt to clarify how the $tRNA^{Lys3}$ transfer from LysRS to RT takes place. Our model suggested that in addition to its interaction with Gag, LysRS might also interact with Pol sequences in order to facilitate this transfer. In fact, as described in Chapter 2, we found a LysRS/Pol interaction, and mapped the binding site for LysRS in Pol sequences to the RNaseH/connection domains in RT. However, an analysis of what the function of this interaction could be does not indicate it is involved in $tRNA^{Lys3}$ transfer from LysRS to Pol. Thus, it had been previously shown that sequences C-terminal of the RT thumb domain in GagPol are not required for $tRNA^{Lys}$ incorporation into virions (296), a process which would require $tRNA^{Lys}$ binding to GagPol. Furthermore, we showed that this LysRS/Pol interaction, which can be seen as a part of a Gag/LysRS/Pol interaction, is not essential for the interaction between Gag and Pol, by demonstrating that reduction of LysRS in this complex, using siRNA to reduce cellular and viral LysRS, did not affect the interaction of Gag with Pol sequences. However, the LysRS/Pol interaction might be involved in the second transfer step, i.e., the transfer of $tRNA^{Lys3}$ from the RT sequences to the PBS. We say this because it has previously been shown that the RT connection domain is required for $tRNA^{Lys3}$ annealing to viral RNA (71). Thus, while deletion of sequences C-terminal of the thumb domain in GagPol will allow for $tRNA^{Lys3}$ packaging into the virus, this tRNA will not be annealed to the viral genome unless the RT connection domain is restored.

Because Gag has been shown to be able to anneal $tRNA^{Lys3}$ to the viral genome (66, 154), we assume that this stage occurs within the $tRNA^{Lys}$ packaging/annealing complex prior to protein processing. In Chapter 3, we investigated the significance of this annealing compared to that facilitated by mature nucleocapsid, NCp7, which will occur after protein processing. This investigation relied on several observations. First, as shown in Figure 3.2C in Chapter 3, the *in vivo* placement of the $tRNA^{Lys3}$ on the viral RNA genome by Gag (using RNA isolated from protease-negative viruses in an *in vitro* $tRNA^{Lys3}$ priming assay) is less than optimal, since, in the *in vitro* $tRNA^{Lys3}$ priming assay using sub-optimal amounts of dCTP (69), the ability during reverse transcription to incorporate the first dNTP, dCTP, is 80% less than that of NCp7-annealed $tRNA^{Lys3}$ (using viral RNA from wild type viruses). In Chapter 3, we also obtained results supporting this observation using a different assay, i.e., a PBS-occupancy test that we developed in the laboratory. In this assay, a DNA oligomer is annealed downstream of the PBS, and then extended by reverse transcription. If $tRNA^{Lys3}$ is annealed to the PBS, the RT-extension of the DNA oligomer will be blocked; if the PBS is open, a longer extension will result. As shown in Figure 3.2 in Chapter 3, this assay appears to work well when viral RNA from protease-positive viruses are used, giving a PBS occupancy of approximately 74%, compared to a 31% occupancy achieved when A3G is present in the protease-positive virus (see below). However, this assay does not appear to work when viral RNA from a protease-negative virus is used. Thus, as shown in Figure 3.1B in Chapter 3, there is only a 20% reduction in $tRNA^{Lys3}$ priming occurring in protease-negative virions compared to protease-positive virions, while the PBS occupancy in protease-negative viruses indicates an 80% drop in PBS occupancy. We interpret this

discrepancy as indicating that the weaker Gag-facilitated annealing of $tRNA^{Lys3}$ in protease-negative viruses results in the RT extension from the DNA primer having the ability to displace the $tRNA^{Lys3}$ from the PBS and produce the longer DNA product.

Second, we have previously shown that A3G has the ability to inhibit $tRNA^{Lys3}$ priming in virions produced from naturally non-permissive cells (H9) and from naturally permissive cells producing A3G from an exogenous plasmid (214). This inhibition had been reproduced in an *in vitro* annealing system using NCp7 to facilitate annealing, and it has been shown that the inhibition of $tRNA^{Lys3}$ priming *in vitro* depended upon the ability of A3G to interact with NCp7 (215). In Chapter 3, we report that while A3G can inhibit NCp7-facilitated annealing, it cannot inhibit Gag-facilitated annealing, and thus we used A3G to further investigate differences between Gag- and NCp7-facilitated annealing in protease-positive and protease-negative viruses, respectively. As shown in this Chapter, the inhibition by A3G of $tRNA^{Lys3}$ priming (Figure 3.1) and PBS occupancy (Figure 3.2) are similar, demonstrating that the reduction in $tRNA^{Lys3}$ priming is a measure of a reduction in $tRNA^{Lys3}$ annealing. In spite of the fact that $tRNA^{Lys3}$ priming from protease-negative viruses is 80% of that from protease-positive viruses, A3G, which doesn't inhibit Gag-facilitated annealing, nevertheless reduces priming in protease-positive viruses to 40% of that found in the absence of A3G. These results do not support a simple two-step model of $tRNA^{Lys3}$ annealing involving Gag-facilitated annealing followed by fine-tuning with NCp7. Rather, they suggest that in protease-positive virions, either the role of Gag in $tRNA^{Lys3}$ annealing is reduced or absent, or exposure of Gag-annealed $tRNA^{Lys3}$ to NCp7 results in a reannealing of $tRNA^{Lys3}$ to viral RNA that is now susceptible to A3G inhibition.

While it is not clear why A3G is unable to inhibit Gag-facilitated $tRNA^{Lys3}$ annealing, it is also not clear how A3G inhibits $tRNA^{Lys3}$ annealing in protease-positive virus. While Gag-facilitated annealing probably occurs prior to viral budding, annealing of $tRNA^{Lys3}$ by NCp7 must occur in the budding or mature virion, and it has been estimated that there are very few A3G molecules present in the virus (approximate 7, +/- 4 molecules A3G/virion (574). Since we have previously shown that A3G inhibition of $tRNA^{Lys3}$ annealing is independent of A3G's cytidine deamination activity, we have considered that A3G may bind to protein in the $tRNA^{Lys}$ packaging/annealing complex that might direct A3G to the annealing site where it could interact with NCp7 involved in annealing. Thus, in the addendum to Chapter 3, we report our observations showing the ability of LysRS to interact with A3G (Figure 3.5). This could occur prior to protein processing within the $tRNA^{Lys}$ packaging/annealing complex, where the Gag numbers are far less than in the immature virion (see discussion in Chapter 2), and in itself might facilitate an interaction of A3G with NCp7 molecules involved in $tRNA^{Lys3}$ annealing. In addition, though we have no supporting evidence for this at present, one can also consider that LysRS may have an ability to bind to stem loop sequences in viral RNA near the PBS. Thus, AsnRS, a synthetase in the same structural class as LysRS (class IIB), has been shown to regulate its own synthesis by specifically binding to a tRNA-like stem loop in the 5′ region of its mRNA, thereby blocking its own translation (172). Whether LysRS can bind specifically to viral RNA in the region of the PBS remains to be tested.

CHAPTER V

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Presented below is a summary of my contributions toward an understanding of the various interactions occurring within the HIV-1 $tRNA^{Lys}$ packaging /annealing complex during my Ph.D. studies at Dr. Kleiman's Lab.

Chapter II: (published in Virology, (479))

In chapter 2, we have shown for the first time that within the $tRNA^{Lys}$ packaging/annealing complex, human lysyl-tRNA synthetase (LysRS), in addition to its interaction with Gag, also interacts with the RT connection/RNaseH domain in Pol. We also showed that this interaction is not required for a Gag/Pol interaction, since over-or underexpression of LysRS had no effect on the Gag/Pol interaction. Based upon other evidence that suggests roles of various structural domains in Pol for $tRNA^{Lys}$ packaging and annealing, we predict that the LysRS/Pol interaction will not be involved in $tRNA^{Lys3}$ packaging, but may be involved in $tRNA^{Lys3}$ annealing.

Chapter III: (submitted manuscript)

The $tRNA^{Lys}$ packaging/annealing complex is composed of the precursor proteins, Gag and GagPol. In chapter 3, we have compared the roles of Gag and NCp7 in the $tRNA^{Lys3}$ annealing process. We showed both in cells and *in vitro* that 1) Gag-annealed $tRNA^{Lys3}$ is annealed more weakly to the viral RNA than $tRNA^{Lys3}$ annealed by mature NCp7, using a newly-developed primer binding site occupancy test as a direct measure of $tRNA^{Lys3}$ annealing. Using this same PBS occupancy assay, we showed for the first time, directly, that A3G inhibits $tRNA^{Lys3}$ annealing. However, we also showed that A3G only inhibits NCp7-facilitated annealing of $tRNA^{Lys3}$, and not Gag-facilitated

annealing. We further used this latter observation to show that annealing in wild-type virus is not a simple two step process, i.e., first annealing by Gag, and then fine-tuning of the annealed complex by NCp7. Rather, NCp7 appears to dominate the annealing process by reannealing the $tRNA^{Lys3}$ in a process that is now sensitive to A3G.

Chapter III: addendum: (unpublished data)

We also demonstrate by coimmunoprecipitation from cell lysates that there exists an interaction between human LysRS and A3G, one that might be crucial for concentrating the few A3G molecules present in a virus to the site of $tRNA^{Lys3}$ annealing.

This work thus provides new insights into the processes that are facilitating the incorporation and annealing of primer $tRNA^{Lys3}$ in HIV-1

CHAPTER VI

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CONTEX ENVIRONNEMENT

certifie que **Jena Saadatmand**
a complété la formation décrite au verso, le 2007/03/26 en conformité avec les exigences du Règlement sur le transport des matières dangereuses

Signature de l'employé Signature de l'employeur
Hôpital général juif Sir. Mortimer B. Davis, LDI

1. Les critères de classification pour la classe 7
2. Les appellations réglementaires pour la classe 7
3. L'utilisation des annexes 1, 2 et 3
4. Les exigences concernant l'obligation de conserver le document d'expédition
5. Les exigences concernant les contenants et les indications de danger- marchandises dangereuses de classe 7
6. Les exigences concernant le plan d'intervention d'urgence applicable à la classe 7
7. Les méthodes à suivre pour la manutention sécuritaire de marchandises de classe 7

Expiration du certificat : 2010/03/26

Lady Davis Institute

PERMIS D'UTILISATION DE RADIOISOTOPE

PERMIS NO. 9

1) RESPONSABLE DU PROJET

Le comité de radioprotection du LDI émet ce permis à:

NOM	Dr Kleiman
LOCAL	227 et 228
CLASSIFICATION	Élémentaire

2) PÉRIODE

Ce permis est émis pour la période suivante: **Début:** 15 février 2008 **Expiration:** 15 février 2009

3) RADIOISOTOPES

A) SOURCES OUVERTES

NATURE	LIMITE DE POSSESSION	LIMITE D'ACHAT
P-32	18,5 MBq (0,5 mCi)	18,5 MBq (0,5 mCi)
S-35	74,0 MBq (2 mCi)	74,0 MBq (2 mCi)

B) SOURCES SCELLÉES

NATURE	ACTIVITÉ	MODÈLE, MARQUE, NO. DE SÉRIE
NIL		

4) LIEUX D'ENTREPOSAGE ET D'UTILISATION

ENTREPOSAGE	227 et 228
UTILISATION	227 et 228

5) PERSONNEL

Les personnes suivantes ont lu le manuel de radioprotection et ont reçu une formation en radioprotection. Elles sont autorisées par le responsable de projet à être présent dans un laboratoire où l'on utilise des substances nucléaires (voir autre page).

INSTITUT LADY DAVIS
POUR LA RECHERCHE MÉDICALE

NOM	PRÉNOM	Utilisateur de substances nucléaires	DOSIMÈTRE PAR TLD OBLIGATOIRE
Yang	Yiliang	Oui	Oui
Wei	Min	Oui	Oui
Niu	Mei Juan	Oui	Oui
Guo	Fei	Oui	Oui
Zhao	Xia	Oui	Oui
Saadatmand	Jenan	Oui	Oui

6) CONDITIONS

1- Une copie de ce permis ainsi que l'affiche info 0728-1 doit être affichée sur les lieux de travail.

2- Toutes les procédures doivent être effectuées en accord avec le manuel de radioprotection du Lady Davis Institute.

3- Le responsable doit s'assurer que seules les personnes autorisées dont le nom apparaît sur ce permis utilisent les sources de radiations mentionnées sur ce permis.

4- Il est interdit de manger de boire ou de fumer dans un lieu susceptible d'être contaminé par du matériel radioactif.

5- Des gants jetables ainsi qu'une blouse de laboratoire doivent être portés lors de la manipulation de substances nucléaires.

6- Le laboratoire doit être verrouillé lorsque non utilisé.

7- Des registres détaillés quant aux achats, à l'utilisation et à l'élimination de radioisotopes doivent être maintenus et disponibles. Il est interdit de transférer des substances nucléaires hors de l'Institut sans autorisation écrite.

8- Les limites de possession indiquées ci-haut sont les quantités maximales pouvant se trouver dans le laboratoire en tout temps.

9- Tous les vols ou pertes de matériels radioactifs ainsi que tous les incidents ou accidents pouvant donner lieu à une exposition aux radiations doivent être portés à l'attention de l'officier de radioprotection et doivent faire l'objet d'un rapport. En tout temps, il est possible de contacter la sécurité pour des urgences.

10- Tous les montages, tous les lieux d'expérimentation et tous les lieux d'entreposage de radioisotopes doivent faire l'objet d'une vérification régulière des niveaux de radioexposition.

11- Les résultats de tous les tests et vérifications (tests de contamination) doivent être consignés dans des registres. Les tests doivent être effectués à chaque semaine ou dans le cas d'utilisation moins fréquente, après chaque utilisation. Les niveaux de contaminations doivent être inférieurs à 3 Bq/cm^2 pour les zones du public pour les substances nucléaires de classe B (conditions généralisées pour le LDI).

12- À l'intérieur du laboratoire, les régions susceptibles de devenir contaminées doivent être clairement identifiées. Les manipulations doivent être effectuées sur des plateaux ou sur les surfaces recouvertes de papier absorbant.

13- Tous les déchets potentiellement radioactifs devront être disposés avec les déchets radioactifs selon les procédures en place à l'institut. Les déchets devront être séparés.

14- Un radiamètre étalonné à tous les ans avec une étiquette de conformité sur l'appareil doit être présent dans le laboratoire pour évaluer les débits de doses et pour vérifier la contamination. Le radiamètre doit être envoyé pour l'étalonnage annuel lorsque l'avis est annoncé.

15- Les utilisateurs du laboratoire doivent porter leur dosimètre en tout temps lorsqu'ils sont dans le laboratoire. Les dosimètres ne doivent pas être entreposés dans le laboratoire lorsque non portés.

Pierre Lavoie

Pierre Lavoie, ing.
Ingénieur physicien, Contex Environnement
Pour,
Institut Lady Davis pour la recherche médicale
3755 Côte Ste-Catherine
Montréal, Québec H3T 1E2
Tel.: (514) 340-8260

Mise à jour : 27 juin 2008

PERMIS D'UTILISATION DE RADIOISOTOPE

PERMIS NO. 9

1) RESPONSABLE DU PROJET

Le comité de radioprotection du LDI émet ce permis à:

NOM	Dr Kleiman
LOCAL	227 et 228

2) PÉRIODE

Ce permis est émis pour la période suivante: **Début:** 1er septembre 2007 **Expiration:** 31 aout 2008

3) RADIOISOTOPES

A) SOURCES OUVERTES

NATURE	LIMITE DE POSSESSION	LIMITE D'ACHAT
P-32	55,5 MBq	55,5 MBq

B) SOURCES SCELLEES

NATURE	ACTIVITÉ	MODÈLE, MARQUE, NO. DE SÉRIE
NIL		

4) LIEUX D'ENTREPOSAGE ET D'UTILISATION

ENTREPOSAGE	227 et 228
UTILISATION	227 et 228

5) PERSONNEL

Les personnes suivantes ont lu le manuel de radioprotection et ont reçu une formation en radioprotection. Elles sont autorisées par le responsable de projet à être présent dans un laboratoire où l'on utilise des substances nucléaires.

NOM	PRÉNOM	Utilisateur de substances nucléaires	DOSIMÈTRE PAR TLD OBLIGATOIRE
~~Desfosse~~	~~Laurie~~	~~Oui~~	Oui
Yang	Yiliang	Oui	Oui
Wei	Min	Oui	Oui
Niu	Mei Juan	Oui	Oui
Guo	Fei	Oui	Oui
Zaho	Xia	Oui	Oui

6) CONDITIONS

1- Une copie de ce permis ainsi que l'affiche info 0728 doit être affichée sur les lieux de travail.

2- Toutes les procédures doivent être effectuées en accord avec le manuel de radioprotection du Lady Davis Institute.

3- Le responsable doit s'assurer que seules les personnes autorisées dont le nom apparaît sur ce permis utilisent les sources de radiations mentionnées sur ce permis.

4- Il est interdit de manger de boire ou de fumer dans un lieu susceptible d'être contaminé par du matériel radioactif.

5- Des gants jetables ainsi qu'une blouse de laboratoire doivent être portés lors de la manipulation de substances nucléaires.

6- Le laboratoire doit être verrouillé lorsque non utilisé.

7- Des registres détaillés quant aux achats, à l'utilisation et à l'élimination de radioisotopes doivent être maintenus et disponibles.

8- Les limites de possession indiquées ci-haut sont les quantités maximales pouvant se trouver dans le laboratoire en tout temps.

9- Tous les vols ou pertes de matériels radioactifs ainsi que tous les incidents ou accidents pouvant donner lieu à une exposition aux radiations doivent être portés à l'attention de l'officier de radioprotection et doivent faire l'objet d'un rapport. En tout temps, il est possible de contacter la sécurité pour des urgences.

10- Tous les montages, tous les lieux d'expérimentation et tous les lieux d'entreposage de radioisotopes doivent faire l'objet d'une vérification régulière des niveaux de radioexposition. En aucun cas le débit de dose en des lieux susceptibles d'être occupés par des étudiants ou des membres du public ou du personnel ne doit excéder 2.5 uSv/hre.

11- Les résultats de tous les tests et vérifications (tests de contamination) doivent être consignés dans des registres. Les tests doivent être effectués à chaque semaine ou dans le cas d'utilisation moins fréquente, après chaque utilisation. Les niveaux de contaminations doivent être inférieurs à 3 Bq/cm^2 pour les zones du public pour les substances nucléaires de classe B (conditions généralisées pour le LDI).

12- À l'intérieur du laboratoire, les régions susceptibles de devenir contaminées doivent être clairement identifiées. Les manipulations doivent être effectuées sur des plateaux ou sur les surfaces recouvertes de papier absorbant.

13- Tous les déchets potentiellement radioactifs devront être disposés avec les déchets radioactifs selon les procédures en place à l'institut.

officier de radioprotection
Lady Davis Institute

Mise à jour : 1er septembre 2007

Commission canadienne de sûreté nucléaire | Canadian Nuclear Safety Commission

Canada

Commission canadienne de sûreté nucléaire

Case postale 1046, Succursale B
Ottawa (Ontario)
K1P 5S9

Tél. : (613) 995-5894 Fax : (613) 995-5086
Pour signaler un incident nucléaire seulement, prière d'appeler l'agent de service 24 heures sur 24.
Tél. : (613) 995-0479

Fiche signalétique de radionucléide

La fiche contient des renseignements sur les radionucléides seulement.
Pour obtenir des renseignements sur les composés chimiques qui incorporent un radionucléide particulier, il faut consulter les fiches signalétiques du SIMDUT.

Partie 1 - IDENTIFICATION DU RADIONUCLÉIDE

Symbole chimique :	P	Nom commun :	phosphore
Masse atomique :	32	Numéro atomique :	15

Partie 2 - CARACTÉRISTIQUES DU RAYONNEMENT

Période radioactive : 14,3 jours
Quantité d'exemption de la CCSN (en Bq) : 1×10^4 (10 kBq)
Un permis de la CCSN n'est pas exigé si la quantité de radionucléides en mains est inférieure à une quantité d'exemption.

Principales émissions	Énergie moyenne (MeV)**	Énergie maximale (MeV)***	Débit de dose à 1 m (mSv/h•GBq)	Blindage recommandé
Neutrons	—	—	—	—
Rayons gamma et rayons X	—	—	—	—
Rayons bêta*	0,6947	1,710	9,17	1 cm de plexiglas
Rayons alpha	—	—	—	—

* La présence de rayonnement bêta générant un rayonnement de freinage (secondaire), un blindage peut donc être requis.
** Énergie moyenne de l'émission la plus abondante.
*** Énergie maximale de l'émission la plus abondante.

Produits de filiation (désintégration)	s/o

Partie 3 - DÉTECTION ET MESURE

Méthode de détection : Détecteur de Geiger-Müller, gammamètres avec des détecteurs à l'iodure de sodium
Dosimétrie :
Externe : DTL (corps entier et peau) ____ DTL extrémités ____ neutrons ____
Interne : corps entier • • thorax ____ analyse d'urine • • autre ____

Partie 4 - MESURES PRÉVENTIVES RECOMMANDÉES

L'acide chromique et ses sels sont corrosifs sur la peau et les membranes muqueuses. Le phosphate de sodium est un irritant léger. Chauffer une solution de Phosphocol ou de phosphate de sodium contenant du ^{32}P jusqu'au point où elle se décompose peut produire des vapeurs radioactives contenant cet isotope.

Vêtements protecteurs recommandés : gants jetables de plastique, de latex ou de caoutchouc; sarrau; lunettes de protection.

Minimisez le temps de manipulation de l'isotope. Pour éviter un contact direct avec la peau, blindez les seringues (avec une feuille d'aluminium ou de plomb) et utilisez des pinces. Si possible, travaillez derrière un écran de plexiglas. Portez une bague-dosimètre si vous utilisez des quantités dépassant quelques dizaines de MBq (~ 1 mCi). Les fioles devraient gainées de lucite.

Optimisez le temps, la distance et le blindage pour minimiser la dose.
Consultez le permis de la CCSN pour connaître les exigences relatives aux contrôles techniques, aux appareils de protection et aux exigences particulières d'entreposage.

Partie 5 - LIMITE ANNUELLE D'INCORPORATION

	Ingestion	Inhalation
Type de composé	Tous les composés	Tous les composés
Limite annuelle d'incorporation (Bq)	8×10^6	8×10^6

CONSIGNES RECOMMANDÉES EN CAS DE DÉVERSEMENT ET DE FUITES

Ces consignes sont destinées aux premiers intervenants. Les mesures indiquées, y compris les mesures correctives, devraient être prises par des personnes qualifiées. En cas de blessure, réelle ou présumée, il faut **en premier lieu** traiter la blessure, et **en second lieu** procéder à une décontamination individuelle.

Techniques de décontamination individuelle
- Lavez bien à l'eau savonneuse toutes les parties touchées; surveillez les réactions cutanées à ces endroits.
- NE FROTTEZ PAS la peau; séchez-la en la tapotant doucement.
- Consultez le permis de la CCSN pour obtenir d'autres détails sur les consignes d'urgence.

En cas de déversement ou de fuite
- Alerter toutes les personnes dans la zone.
- Confiner le déversement ou la fuite à l'aide d'un matériau absorbant.
- Faites évacuer la zone.
- Appelez sur les lieux le préposé aux urgences ou toute autre secouriste disponible.

Exigences minimales en matière d'équipement de protection
- Gants
- Protège-chaussures
- Lunettes de protection
- Survêtement ou autre vêtement de protection facile à retirer.
- Respirateur convenable
- Consultez le permis de la CCSN pour obtenir d'autres détails.