IMPORTANCE OF tRNA$^{\text{Lys},3}$ STRUCTURE AND USE IN GAG TRANSLATION FOR PRIMER SELECTION REQUIRED FOR REPPLICATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE I

by

WANFENG YU

CASEY D MORROW, COMMITTEE CHAIR
PETER BURROWS
CHENBEI CHANG
JIM COLLAWN
JEFFREY ENGLER

A DISSERTATION
Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BIRMINGHAM, ALABAMA
2007
IMPORTANCE OF tRNA\textsuperscript{Lys,3} STRUCTURE AND USE IN GAG TRANSLATION FOR PRIMER SELECTION REQUIRED FOR REPLICATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE I

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ABSTRACT

The features of tRNA\textsuperscript{Lys,3} that dictate why human immunodeficiency virus exclusively selects this tRNA as the primer for initiation of reverse transcription is unknown. The post-transcriptional modification at nucleotide 58 in the TΨC stem-loop could play a role during plus-strand synthesis to stop reverse transcriptase from re-copying the tRNA primer. Nucleotides 53 and 54 within the TΨC stem loop of the tRNA have been shown to be important to form the complex between tRNA and the HIV-1 viral genome during initiation of reverse transcription. To further delineate the features of the TΨC stem loop in tRNA\textsuperscript{Lys,3} in reverse transcription, we used a complementation system in which \textit{E.coli} tRNA\textsuperscript{Lys,3} is provided in trans to a mutated HIV-1 genome in which the PBS is complementary to this tRNA to ascertain the effects that different mutants in the TΨC stem loop of tRNA\textsuperscript{Lys,3} have on complementation. Alteration of nucleotide 58 from A to U (A58U), T54G and TG5453CC all resulted in tRNA\textsuperscript{Lys,3} that was aminoacylated when expressed in cells, while a T54C mutation resulted in a tRNA\textsuperscript{Lys,3} that was not aminoacylated. Both the A58U and T54G mutated tRNA\textsuperscript{Lys,3} complemented HIV-1 replication similar to wild type \textit{E.coli} tRNA\textsuperscript{Lys,3}, but the TG5453CC tRNA\textsuperscript{Lys,3} mutant did not complement replication, thus, post-transcriptional modification of nucleotide 58 in tRNA\textsuperscript{Lys,3} is not essential for HIV-1 reverse transcription. In contrast, nucleotides 53 and 54 of tRNA\textsuperscript{Lys,3} are important for aminoacylation and selection and use of the tRNA\textsuperscript{Lys,3} in reverse transcription.
My research also focused on the link between the process of primer selection and lysine codon use in Gag. Proviral genomes were created in which the five codons specific for lysines prior to the Gag-pol junction were altered to be specific for tRNA\textsubscript{Lys,3} or tRNA\textsubscript{Lys1,2}. Greater amounts of infectious virus were produced using the \textit{in trans} complementation system in which the five codons were specific for tRNA\textsubscript{Lys,3} compared to the wild type or genomes with the five codons specific for tRNA\textsubscript{Lys1,2}. To extend this work, viral genomes were created in which all of the lysine codons except the five lysine codons prior to the Gag-pol junction were modified to be specific for tRNA\textsubscript{Lys1,2}. Analysis of the production of infectious virus from transfection of these proviral genomes revealed lower levels of virus regardless of whether the five codons preceding the Gag-pol frameshift were specific for tRNA\textsubscript{Lys,3} or tRNA\textsubscript{Lys1,2}. These results demonstrate that structure features of tRNA\textsubscript{Lys,3} and lysine codon preference within the Gag protein of HIV-1 have profound impact on the production of infectious virus and lead support to the idea that there is a link between viral translation and the process by which HIV-1 selects the tRNA primer used for reverse transcription.
ACKNOWLEDGMENTS

First and foremost I thank my mentor, Dr. Casey Morrow, for his support and guidance throughout my graduate career. I have learned a lot of skills necessary for a successful career from him. I sincerely appreciate his kindness and patience.

I thank the members of my thesis committee, Drs. Peter Burrows, Chenbei Chang, Jim Collawn, and Jeffrey Engler. They have been very supportive and have made many helpful suggestions during the years.

Thanks also go to members of Dr. Morrow’s lab, past and present, for their support and friendship along the way. I have learned a great deal from them.

Also, I acknowledge my parents, Qiying Yu and Pengyuan Wan, for their immeasurable love and all the hardship they went through to support me. In addition, a word of gratitude goes to my sister and brother-in-law. They have been very supportive and encouraging.

I especially thank my wonderful husband, Yun, for his undying support and love. He has shared the many challenges and stress for me during my graduate career. His love, patience and encouragement accompanied me through all years. My thanks also go to my parents-in-law for their love and supports.
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<td>nucleotide containing an adenine base</td>
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<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
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<tr>
<td>A-loop</td>
<td>adenine-rich loop</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>C</td>
<td>nucleotide containing a cytosine base</td>
</tr>
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<td>CA</td>
<td>capsid</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Env</td>
<td>retrovirus envelope glycoprotein</td>
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<td>G</td>
<td>nucleotide containing a guanine base</td>
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<td>retrovirus gag gene-encoded precursor polyprotein</td>
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<td>IN</td>
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<td>kDa</td>
<td>kilodalton</td>
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<td>long terminal repeat</td>
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<td>MA</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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LIST OF ABBREVIATIONS (Continued)

NC  nucleocapsid
Nef  negative factor protein
N-terminal  amino-terminal
PAS  primer activation site
PBS  primer binding site
PPT  polypurine tract
PR  protease
R  the repeat region of the retrovirus genome
RNA  ribonucleic acid
RNase  ribonuclease
RRE  Rev-response element
RT  reverse transcriptase
SIV  simian immunodeficiency virus
SU  surface domain of Env
TAR  trans-activating response element
Tat  trans-activating response element
tRNA  transfer RNA
U  nucleotide containing a uracil base
U3  the 3’unique region of a retrovirus genome
U5  the 5’unique region of a retrovirus genome
Vif  virion infectivity factor
-sssDNA  minus strand strong-stop DNA
LIST OF ABBREVIATIONS (Continued)

<table>
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<td>+sssDNA</td>
<td>plus strand strong-stop DNA</td>
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<tr>
<td>Ψ</td>
<td>pseudouridine</td>
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INTRODUCTION
Overview

Human immunodeficiency virus is the causative agent of AIDS. The world’s first known case of AIDS has been traced to a sample of blood plasma from a man who died in the Democratic Republic of Congo in 1959 (1). A genetic analysis of HIV in 2003 indicates that it may have first arrived in the United States in 1968. The first documented cases of AIDS are from New York and California in 1981 (1). The next year, scientists in Centers for Disease Control and Prevention (CDC) predicted that the immune system disorder affecting gay men was due to an infection and established the term Acquired Immune Deficiency Syndrome (AIDS). The virus responsible for AIDS was independently discovered by Luc Montagnier of the Pasteur Institute in Paris, France, and Robert Gallo of the National Cancer Institute in Washington DC, US. It is later named the human immunodeficiency virus (HIV). WHO/UNAIDS estimates that 39.4 million people were living with HIV-1 infection by December of 2004, with 5 million new infections each year (1).

Currently, there is no effective cure for AIDS. Various drugs targeted to the different steps of viral replication have been declared, but drugs such as soluble CD4 or dextran derivatives aimed to inhibit or interfere with the gp120-CD4 attachment step have shown little or no clinical benefit. Protease inhibitors or interferons acting at the post-transcriptional level are currently under phase I and II investigation. The only group of
compounds clinically active belongs to the nucleoside analogues that act as DNA chain terminators and by inhibiting viral reverse transcriptase. Zidovudine (AZT), dideoxynosine (ddI) and dideoxycytidine (ddC) have been extensively studied, and used on a large clinical scale. Stavudine (D4T), deoxyfluorothymidine (FLT) and 3'thiacytidine (3TC) are entering phase I-II studies. Being the first nucleoside analogue discovered and used since early 1985, zidovudine remains the gold standard of anti-HIV therapy. Despite 20 years of effort, an effective vaccine for HIV-1 is still a long way off. The biggest obstacle in HIV-1 vaccine development is perhaps the extremely adaptive and genetically variability of this virus. The high mutation rate of the HIV-1 genome makes it exceedingly difficult for successful vaccination and elimination of virus. Further understanding the details of the HIV-1 replication process during infection may show that it is possible to abolish virus infection by blocking its pathway of replication in cells.

Classification of HIV

Two strains of HIV, designated HIV-1 and HIV-2, have been identified in association with AIDS. Of these 2 forms, HIV-1 is the more prevalent and the primary pathogen isolated in AIDS patients worldwide; it accounts for 80% of HIV infections in the United States (2). HIV-2 appears to be most prevalent in the nations of western Africa and it is less virulent overall than HIV-1 (3). The genome of HIV-2 has a homology of 70% with the genome of SIV, and 40% with HIV-1. It has been hypothesized that given this genetic similarity with SIV, HIV may have been recently introduced into the human population, crossing over from primates into humans (7).
According to the phylogenetic sequence differences in the envelope nucleotide sequences, HIV-1 is classified into group M (for “main”), O (for “outgroup”) and N (Non M/O) globally. Group M is responsible for the majority of infections worldwide; group O is a relatively rare group currently found in Cameroon, Gabon, and France. Group N represents viruses found in only a few individuals living in Cameroon (6). Group M can be further divided into at least eight distinct subtypes or clades (A through J) (4-5). Among these subtypes, clade C predominates globally, and clade B predominates in North America and Europe.

HIV-1 Genetic Organization and Viral Proteins

HIV-1 belongs to the retroviridea, and contains an RNA genome of approximately 9.2Kb in length, which contains nine open reading frames, including gag (group specific antigen), gag-pol (protease reverse transcriptase integrase) and env (surface glycoprotein envelope). The genome also encodes three regulatory proteins: Rev (regulator of virion protein), Tat (transactivator of transcription), and Nef (negative regulatory factor). Three other accessory proteins that are probably involved in virus maturation and release are encoded: Vif (virion infectivity factor), Vpu (viral protein U), and Vpr (viral protein R).

LTR

Two long terminal repeats (LTR) consisting of a U3-R-U5 segment are located at both the 5’ and 3’ end of the HIV-1 integrated proviral genome. U3 termed unique sequence of 3’end, U5 termed unique sequence of 5’end, and R termed repeat
Fig. 1. Schematic cross section through an HIV-1 virion. Major components of the virion are illustrated.
Fig. 2. Genetic organization of HIV-1 RNA genome. The boxes represent the relative positions of the nine open-reading frames. Major RNA elements in the genome are shown in the approximate locations.
sequence. The 5’ non-translated region of the genomic RNA is very important for viral replication. The first 60 nucleotides of the 5’ end, termed as the TAR region, are essential for viral gene expression. The PBS (primer binding site), which is located at nucleotides 183-201, is a critical region of the viral RNA genome for initiation of reverse transcription. The PBS is complementary to the 3’ terminal 18 nucleotides of tRNA\textsubscript{Lys}, which is used to initiate reverse transcription. One of the HIV-1 encapsidation signals (\(\psi\)) is located between the splice donor and initiation codon for Gag. Like other retroviruses, HIV packages two RNA genomes that are tightly associated through numerous interactions. And the dimerization initiation site (DIS) (nucleotides 235-281) was recently suggested to be important in RNA packaging and possibly the second strand transfer during reverse transcription.

The R-U5-PBS region of the HIV-1 proviral DNA has been shown to contain numerous binding sites for transcription factors (8). In particular, AP1 (nucleotides 155-163) and NFAT/AP3L (nucleotides 165-178) sites have been mapped immediately upstream of the PBS, while an IRF/DBF and two Sp1 sites have been mapped downstream of the PBS prior to the start of Gag. The effects of the mutations alone or in combination on virus replication were analyzed in continuous cell lines or primary T-cells. Single mutations in the AP1 and AP3L/NFAT elements did not affect replication in continuous or primary cells. Mutation in the downstream Sp1 sites does not affect transcription. Extensive secondary structure in the 5’ untranslated leader region of the HIV-1 genome has been suggested by electron microscopy, replication studies with mutant viruses, and biochemical RNase probing studies (184-189). This research, combined with phylogenetic analyses and computer assisted structure prediction, led to a model of the secondary RNA
structure of the complete leader region of the HIV-1 genome (190). According to this model, the PBS is flanked by an upstream small stem-loop structure, the U5-PBS hairpin. A striking feature of the U5-PBS hairpin is that part of the PBS sequence is involved in base pairing. Beerens et al (191) demonstrated the importance of U5-PBS hairpin structure for efficient HIV-1 replication. Both stabilization and destabilization of this RNA structure decreased the viral replication capacity.

A further complication for studies in this region of the HIV-1 genome is that there have been reports that the 5’NTR functions as an internal ribosome entry site (IRES) (9). The implications of the finding for HIV-1 translation are not clear. However, previous studies with picornavirus IRES have clearly shown that RNA structures are important for activity. Thus, mutations within the 5’NTR would undoubtedly influence the RNA structure and possibly IRES function. Collectively, the results of studies on reverse transcription, transcription, and translation point to a complexity in the R-U5-PBS region of the HIV-1 genome that is an important topic in understanding HIV-1 replication.

**Gag**

The *gag* gene of HIV-1 encodes structural proteins required for virion assembly. The *gag* gene is translated as a polyprotein precursor (Pr55*Gag*) consisting of the matrix (MA) protein, capsid (CA) protein, and nucleocapsid (NC) protein (10). The Gag polyprotein is cotranslationally modified by the attachment of myristic acid to its amino-terminal glycine residue immediately after removal of the terminal methionine (11). The myristylation of the Gag polyprotein is required for the assembly and release of particles
from infected cells, since substituting an alanine for a glycine resulted in a nonmyristylated Gag precursor and production of a noninfectious virus (11, 12).

The 17kDa MA protein, which is released from the Pr55Gag precursor, is associated with the inner surface of the virion phospholipid bilayer and with the core structure. It lines the inner surface of the virus envelope and is a component of an envelope associated icosahedral capsid (13). Studies also suggest that MA interacts with the Env proteins during budding. By the use of nuclear magnetic resonance (NMR), the three-dimensional structure of HIV-1 MA protein in solution was determined (14, 15). In solution, MA is a monomer consisting of five helices, which are connected to each other by short loops or β-strands, with four of the helices surrounding the fifth one to form a hydrophobic core. Trimeric in the crystal, MA protein contains a globular amino-terminal domain and a smaller carboxy-terminal domain projecting away (16).

The CA protein has a molecular mass of 24 kDa and is approximately 200-270 amino acid residues in size. It constitutes the shell of the cone-shape core structure in the mature virion containing genomic viral RNA and viral enzymes. CA is largely helical, and the helices in the CA fragment pack together to form an arrowhead shape, with the carboxy-terminal end at the tip and the amino-terminal end near the base (16). The amino-terminal sub domain and carboxy-terminal sub domain are connected by a flexible linker (18). The amino terminus itself, which contains seven α-helices and two β-hairpins (19-22), is folded back so that it is buried into the molecule. The carboxy sub domain is composed of four α-helices and forms a relatively stable dimer in solution (23-24). The CA protein is suggested to play a role in virus assembly (17). Numerous molecular genetic analyses have been carried out to explore the function of CA in assembly.
The NC protein, positioned near the carboxy terminus of the Gag polyprotein, is a small basic protein, typically about 60-90 amino acid residues long. The NC protein is tightly associated with viral genomic RNA within the core of the virion. It has two characteristic motifs made of regularly spaced cysteine and histidine residues, which contain the structure CX$_2$CX$_4$HX$_4$C (abbreviated CCHC). One of these motifs appears to have the paramount role in RNA encapsidation. The CCHC motif is called a “zinc finger”, which coordinates a Zn$^{++}$ ion and binds certain proteins to nucleic acids. Studies have shown that NC binds Zn$^{++}$ ion tightly both in vitro and in the virion (25-26). In the presence of Zn$^{++}$, the two CCHC motifs fold into a compact structure; in contrast, the amino-terminal and carboxy-terminal portions of the polypeptide appear to remain flexible. Mutations that disrupt the CCHC motif impair packaging of viral RNA genomes into virion particle (27). NC also contains sequences which are required for virion assembly or budding.

The p6 protein, derived from the carboxy terminus of the Gag polyprotein, is a 6kDa proline-rich peptide. The p6 is considered to be associated with virion release from the cell surface since the absence of the p6 sequence leads to the accumulation of virus particle assembled at the cell membrane without efficient release (28).

**Pol**

The *pol* gene of HIV-1 encodes three functional enzymes that are necessary for virus replication: protease, RT, and integrase. The *pol* gene is translated from the same genome length mRNA as the *gag* gene. The open reading frame for the *pol* genes overlaps the open reading frame for the *gag* by 241 nucleotides, with *pol* in the -1 translation-
al reading frame with respect to \textit{gag}. The expression of the \textit{pol} gene of HIV-1 occurs through a process called “frame shift”, where the ribosome “slips back” one base during translation, resulting in the synthesis of a fusion protein between the Gag and Pol proteins (Pr160\textsubscript{gag-pol}) (29-30). Studies using cell-free translation systems have shown that the RNA sequence where the frameshifting occurs contains a homopolymeric sequence of six uridines followed by a hairpin loop structure, which is suggested to play an essential role in ribosomal frameshifting. The frequency of ribosomal frameshifting has been estimated to be around 10%, resulting in a Gag to Gag-pol ratio of around 9:1 (12).

As a result of autoprocessing event from Pr160Gag-pol (31-34), the protease is responsible for specific cleavage events leading to the release of the mature protease, RT, and integrase from the polyprotein. The proteolytic processing by the viral protease is necessary for replication of infectious HIV particle.

The RT of HIV-1 is encoded from the Pr160Gag-pol polyprotein and is subsequently processed by the pol-encoded protease to yield the active form of the enzyme. This enzyme has multiple catalytic activities, including a DNA polymerase activity that catalyzes either RNA-dependent or DNA dependent polymerization (35-36) and a ribonuclease H (RNase H) activity that specifically degrades the RNA strand of an RNA-DNA hybrid duplex (37-38). The mature RT is a heterodimer composed of two subunits, p66 and p51. Both p66 and p51 subunits contain a polymerase domain composed of four sub-domains called fingers, palm, thumb, and connection. These sub-domains are arranged quite differently in the two subunits, with the small subunits more tightly packed than the large subunits, even though the amino acid sequence of p51 is identical to the amino-terminal portion of p66 (16). P66 exhibits both DNA polymerase and RNase H
activity, whereas p51, lacking the carboxy-terminal sequence compared to p66, exhibits neither activity, making the role of p51 in the reaction unclear (39-40).

A second protein derived by proteolytic processing of the Pr160 Gag-pol polyprotein is integrase. This 32kDa protein is essential for the integration of the viral DNA into the host cell chromosome (41-42). The enzyme consists of three domains. The N-terminal domain has a His2Cys2 motif which chelates zinc, the core domain has the catalytic DDE motif which is required for its enzymatic activity, and the C-terminal domain has an SH3-like fold which binds DNA nonspecifically (43). Purified integrase is able to recognize the ends of the newly synthesized linear double-stranded viral DNA, to remove two nucleotides from the 3’ end of each strand, and to connect this DNA end to a target DNA at essentially random sites.

Env

The HIV-1 Env glycoprotein is synthesized as an 88kDa precursor protein from a spliced version of viral genomic RNA. The precursor contains the hydrophobic amino-terminal signal sequence, which serves to direct the protein into the secretory pathway of the host cell. During translocation into the rough endoplasmic reticulum, the hydrophobic amino-terminal signal sequence is cleaved, and the precursor is glycosylated and folds into an appropriate tertiary structure (44). It then undergoes oligomerization, which is important for intracellular transport and stability (45-47). The glycosylated 160KDa envelope precursor is cleaved in the Golgi by a cellular protease to produce a 120kDa SU protein and a 41kDa TM protein.
The SU protein interacts with host cell receptor CD4 (48-49) and determines the ability of the virus to infect cells of the monocyte/macrophage lineage (50-53). SU protein is composed of five variable regions (V1-V5) and four constant regions (C1-C4). The variable regions and constant regions are interspersed to each other. V1-V4 are believed to form loops that extend outward from the central domain of SU. Among them, V3 formed variable loop is a major determinant for chemokine receptor binding, which make V3 a major target for neutralizing antibodies.

The TM protein of gp41 contains complex sugar side chains and a stretch of hydrophobic amino acids which enable the protein to anchor on the cell membrane and to catalyze fusion between the virus membrane and the target cell membrane (54-56). Triggered by SU protein binding to CD4 and a coreceptor, conformation of the TM protein shifts from a native prefusogenic state to a fusogenic state, in which the N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR) associate to form a six-helix bundle, representing the fusion-active gp41 core (57). Any compound that disrupts the gp41 six-helix bundle formation may inhibit the gp41-mediated membrane fusion, thereby blocking HIV-1 entry into target cells. Thus, TM protein is another attractive target for anti-virus therapy.

Viral accessory genes

Rev. Rev is an 18kDa protein translated from a small multiple spliced mRNA. It binds to a cis-acting RNA target, the Rev response element (RRE), which is present in all unspliced viral transcripts. Rev consists of an arginine-rich NH$_2$-terminal domain that mediates RRE binding as well as nuclear localization and a COOH- terminal leucine-rich
domain that contains a nuclear export signal (58). The nuclear localization signal and nuclear export signal each function by joining into cellular pathways for nuclear import and export. The NMR-based studies revealed that four arginine residues (at positions 35, 39, 40 and 44) participate in base-specific contacts with the high-affinity binding site in stems IIB and IID of the RRE, whereas other residues (the threonine at position 34 and the arginines at positions 38, 41-43, 46, and 48) contact the sugar-phosphate backbone (59-60). However, the contributions of these amino acids in Rev function have not been fully evaluated since substitution of each of these specific residues does not inhibit Rev activity in vitro (60-61).

Tat. Among the six regulatory or accessory HIV-1 genes in addition to gag, pol, and env, tat was one of the first to be identified. A 14kDa viral protein composed of 86 amino acids, tat protein activates high level transcription of integrated viral DNA from the host cell chromosome. The domain structure of Tat includes an activation domain and a RNA binding domain. Tat functions as a transcriptional activator through interaction with an RNA stem-loop structure, TAR, which is present at the 5’terminal of viral mRNA. Different from most other transcriptional activators, Tat acts mostly at the level of transcriptional elongation rather than initiation (62). A cellular protein kinase complex called TAK (Tat-associated kinase) binds specifically to the activation domain of Tat and can phosphorylate the COOH-terminal domain (CTD) of RNA polymerase II (63), a step implicated in regulation of transcriptional elongation. Tat has been found to induce neuronal dysfunction/toxicity, resulting in CNS pathology (64).

Nef. Nef is a 27-35kDa, membrane-associated, N-terminally myristylated phosphoprotein. Nef is suggested to have multiple functions. One important function is the
down-regulation of the cell surface CD4 receptor. CD4 down-modulation could allow more Env protein to be incorporated into assembling of viral particles (65) and prevent the super-infection of the host cell. Additionally, Nef has been shown to increase transcription and processing of cholesterol molecules; it is also involved in the transportation of newly synthesized cholesterol to membrane lipid rafts (66). Virions assembled with wild-type levels of cholesterol have been found to be more infectious in a CD4-independent pathway especially in primary T cells than Nef defective mutants (66). Finally, Nef plays a role in the down-regulation of the major histocompatibility class (MHC-I and MHC-II) molecule presentation, which enables the virus to avoid immune detection (67-70).

**Vif.** The function of the small accessory protein was largely unknown. This highly basic, 23kDa, phosphorylated protein is translated from a single spliced Rev-dependent RNA. Discovery of the cellular antiviral protein, human apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like-3G (APOBEC3G) (71-76) has cast light on the main function of Vif. APOBEC3G is a cytidine deaminase that is incorporated into HIV-1 virions from virus-producing cells and results in the deamination of cytidine (C) to (U) of minus-strand reverse transcripts after infection. These minus-strand hypermutations result in the inhibition of viral infectivity and replication of HIV-1 virus (72). Vif functions to block the APOBEC3G and directs it to ubiquitin pathway for degradation by the proteosome and as a result is excluded from virions (77). The complete mechanism of Vif inhibition of APOBEC3G activity is unknown yet.

**Vpu.** Vpu is a unique accessory protein that is exclusively found in HIV-1, not in HIV-2 and most SIV (with the exception of SIV cpz and SIV gsn) (78-79). This 16kDa
viral integral membrane spanning protein contains an N-terminal hydrophobic region, followed by two highly conserved constitutively phosphorylated serine residues, linked to a negatively-charged amino acid segment that extends into the cytoplasm (82-84). It serves two independent functions in the HIV-1 life cycle. First, it binds to CD4 in the ER and targets it for proteolysis through the ubiquitin-proteasome pathway (80). It has been demonstrated that Vpu binds to a protein termed β-TrCP, which binds to the proteasome targeting factor Skp1p. This interaction was confirmed by the isolation of CD4-Vpu-β-TrCP ternary complexes in vivo and by the fact that Vpu induced degradation of CD4 was blocked by a mutant form of β-TrCP that was unable to bind Skp1p (81). The second function of Vpu is to help the release of viral particles from an infected cell by counteracting host restriction factors (85-89). Recently, Vpu has been discovered to inhibit nuclear factor Kappa B (NF-κB) activation (90). Together, the functions of Vpu are important for the virion assembly and release of infectious virus particles.

Vpr. Vpr is a 14kDa virion associated protein. It is incorporated into the virion due to the interaction between C-terminal domain of Vpr and C-terminal portion of the Gag precursor protein, specifically in the mature nucleocapsid and p6 region (91-95). Vpr has been demonstrated to mediate the nuclear import of HIV-1 PICs (preintegration complex) into the nucleus of nondividing cells, such as primary macrophages (96). Another distinct role of Vpr is to induce cell-cycle arrest in the G2/M phase as well as to activate transcription of HIV-1 LTR by associating with multiple transcription factors, such as SP1 and TFIIB (97-98). Vpr also plays a role in inducing T cell apoptosis (99).
The HIV-1 Replication Cycle

The life cycle of HIV-1 involves a series of steps important for the efficient infection of human target cells. After fusion of the viral membrane and membrane of the target cell, the viral RNA genome enters into the host cell cytoplasm, where it is converted to a DNA form through the process of reverse transcription, followed by integration of the DNA genome into the host cell chromosome DNA. The proviral DNA is transcribed by cellular RNA polymerase II, transcripts are transported out of the nucleus and translated into viral proteins. Together with progeny RNA, viral proteins are assembly at the cell periphery and plasma membrane. During a process termed budding, the viruses are released outside the host cell and subsequently undergo maturation.

Entry

There are two basic pathways for membrane enclosed viruses to enter host cells, pH-dependent endocytosis and direct fusion with the plasma membrane. HIV-1 primarily uses receptor-mediated fusion at the plasma membrane in order to enter host cells. In some cases, it appears that HIV-1 can also enter by an alternative endocytic pathway; however, this usually results in inactivation or degradation in lysosomes (100). The process of HIV-1 target cell recognition is defined by viral gp120 and gp41 interacting with the cell surface receptor, CD4, as well as coreceptors from certain members of the chemokine family, including CCR5 and CXCR4. CD4, a 60kDa molecule, was originally described as a cell surface marker for the helper subset of mature T lymphocytes. As a coreceptor for MHC-II, CD4 plays an important role in immune recognition and T-cell activation (101). The viral entry process consists of receptor binding followed by
Fig. 3. HIV-1 replication cycle. Major steps are described in the text.
coreceptor binding, and ultimately membrane fusion allowing the viral core to enter the cell. First, the viral gp120 recognizes CD4 on cell surface. This interaction causes conformational changes in gp120, and exposes coreceptor binding sites. Binding of coreceptor CCR5 or CXCR4 to the gp120-CD4 complex enables the viral membrane to attach to the cellular membrane (102-104). It also triggers a conformational change of HIV-1.
gp41 ectodomain. As a result, the fusion peptide of gp41 is inserted directly into the membrane and form fusion pore between the two layers of the target membrane and membrane fusion occurs. It has been estimated that membrane fusion requires the cooperation of multiple CD4 receptors, four to six CCR5 coreceptors, and three to six Env trimers (105-106). Completion of membrane fusion allows the viral core to enter the target cell by a process termed uncoating (107). Once the viral core is released into the cytoplasm of the cell, it is faced with the challenge of delivering its genetic material to the nucleus. Studies have shown that the HIV-1 core transports its genome towards the cell nucleus through microtubules (108).

**Reverse transcription**

The process of converting single–stranded genomic RNA into double-stranded proviral DNA is termed reverse transcription, which is a central step in the HIV-1 life cycle. This process is mediated by the virus encoded enzyme reverse transcriptase. The general model of reverse transcription includes two obligatory DNA transfer reactions. HIV-1 reverse transcriptase utilizes host-encoded tRNA<sub>Lys,3</sub> as primer. Annealing of the tRNA primer to the viral plus-strand RNA takes place in a region approximately 200 nucleotides downstream from the 5’end of the unspliced full length RNA, at a location termed as the primer binding site (PBS). PBS-annealed tRNA<sub>Lys,3</sub> is extended from its free 3’ hydroxyl by RT to initiate the generation of a minus-strand strong stop DNA (−sssDNA). Following RNase H mediated degradation of the RNA strand of the RNA:−sssDNA duplex, the first strand transfer cause −sssDNA to be annealed to the 3’end of a viral genomic RNA. This transfer is mediated by identical sequences known as the re-
peated (R) sequences, which are present at the 5’ and 3’ end of the RNA genome. The 3’ end of -sssDNA was copied from the R sequences at the 5’ end of the viral genome and therefore contains sequence complementary to R. After the RNA template has been removed, -sssDNA can anneal to the R sequence at the 3’ end of the RNA genome; and synthesis of –sssDNA resumes, accompanied by RNase H digesting the template strand. However, the RNA genome contains a short polypurine tract (PPT) that is highly resistant to RNase H degradation. PPT primes synthesis of plus-strand DNA. Plus-strand synthesis is paused exactly after the primer binding site sequence, the position at the tRNA 58A, a methylated adenosine suggested to be the termination signal of plus-strand DNA synthesis, yielding a DNA termed plus-strand strong-stop DNA (+sssDNA). Ben-Artzi et al (112) reported two determinants that may serve as stop signals for +sssDNA synthesis. One stop signal was the methylated A58 residue in tRNA^Lys,3. The second stop signal was the secondary structure of the PBS sequence, which appeared to contribute to stronger termination in an in vitro system. RNase H removes the primer tRNA, exposing sequences in +sssDNA that are complementary to the PBS sequence at the 3’ end of minus-strand DNA. Annealing the complementary PBS segments of both strands DNA constitute the second strand transfer and complete the synthesis of plus strand (16).

Thus, the PBS plays a dual role in reverse transcription, i.e., by providing an anchor for the tRNA primer at the initiation of the reaction and by facilitating the second-strand transfer. The latter is presumably an intramolecular event that involves formation of a circular intermediate (109-110). The consequence of the two strand transfer reactions is that the provirus acquires a duplicated U3-R-U5 sequence, termed long terminal
repeats at both of its ends (111). Reverse transcription finally produces a double strand DNA product that is longer than the original genomic RNA.

**Nuclear import**

After reverse transcription, the viral double stranded-DNA in the PIC (pre-integration complex) needs to be transported the cytoplasm into the nucleus for integration into chromosomal DNA. Once near the nuclear membrane, HIV-1 most likely relies on the cellular nuclear import proteins to pass through the nuclear pore (113). An alternative mechanism based on the function of Vpr to induce reversible ruptures of the nuclear membrane, which might serve as entry points for the PIC, has been suggested (114). However, this hypothesis has not been confirmed experimentally in the context of HIV infection.

The model most favored by investigators working in the area of HIV nuclear import is that the HIV PIC itself is karyophilic. In this model, a component or components of the PIC contain targeting signals that recruit cellular transport proteins, which then direct the PIC passage through the nuclear pore. Three viral protein, MA, IN, and Vpr, have been suggested as karyophilic agents that engage the cellular nuclear import machinery to the PIC. MA was the first viral protein discovered to be involved in HIV-1 nuclear import (115,116). MA molecules are found in tight connection with the HIV core and the PIC (117,118). HIV-1 MA carries two functional, albeit rather weak though, nuclear localization signals (NLSs) (119), which contain a short stretch of basic amino acids that introduce a positive charge that is crucial for the nuclear targeting properties of these sequences (120). Mutations of this area in MA significantly decrease HIV-1 repli-
cation in nondividing cells (121). The possibility that IN is involved in HIV-1 nuclear import is suggested by the fact that IN is a highly karyophilic protein itself and can interact with importin α/β, which was thought to be independent in the process of nuclear import (122). NLS spanning residues 161-173 has been identified within the central core domain of IN (123). Mutations within this domain, such as V165A or R166A, disrupted the of nuclear import activity of integrase (113). Another HIV-1 encoded protein that has been implicated in nuclear import is Vpr. One hypothesis for the role of Vpr in HIV-1 nuclear import is that Vpr targets the HIV-1 PIC to the nucleus via a distinct, importin-independent pathway, suggesting that Vpr might mediate binding of the PIC to the nuclear pore, thus performing a function normally attributed to importins (124-127). Vpr is also suggested to bind on importin α and change its affinity for the nuclear localization signal (125,128). In either case, Vpr appears to take part in the process of HIV-1 nuclear import, particularly in macrophages.

Integration

The process of integration of viral DNA into the host chromosome is catalyzed by IN and the resulting integrated viral DNA is termed as provirus. This process has been defined to be a two-step reaction. First, IN cleaves two nucleotides from each 3’ terminus of the double –stranded viral DNA (129), exposing the 3’OH groups and allowing the second step strand transfer to occur. The recessed 3’OH viral DNA ends are then ligated to the 5’-O-phosphate ends of target DNA strands, which n cut, at the site of integration. Energy for integration is provided by the cleavage of the phosphodiester bond in the host cellular DNA. Finally, cellular enzymes presumably repair gaps at each junction,
completing the integration process (130). The site of integration in the chromosomal DNA is not consensus. It is generally believed that integration preferentially occurs in regions that are highly transcribed by RNA Pol II (131) or regions that are not blocked by DNA binding proteins, such as histones (132).

Transcription, processing and nuclear export

After integration, the viral life cycle enters into the late phase. Transcription of viral genes is mediated by cellular polymerase II, along with cellular mRNAs and snRNAs. LTR region contains most of the transcriptional control sequences, act as promoter and enhances, that attract cellular transcriptional factors. Regulatory elements important for RNA polymerase II, such as SP-1, TATA box, and chicken ovalbumin upstream promoter, are located in the U3 region of the LTR (133-137). DNA transcription enhancer located in the 5’DNA U3 include NF-kB, NF-AT, USF-1 (upstream stimulatory factor-1), as well as sequences for leader binding protein-1 (LBP-1) and CCAAT-binding transcription factor (CTF)/nuclear factor (NF-1) (138-141). The basal transcriptional activity of LTR alone is very low, which need transcriptional activator, Tat, to shift gene transcription to higher level by promoting the recruiting of transcriptional active complex.

After viral genome transcription, there types of mRNA are expressed from the provirus: multiply spliced mRNA encoding Tat, Rev and Nef; partially-spliced mRNA that is translated into Env, Vif, Vpr and Vpu proteins; unspliced mRNA encoding Gag and Gap-Pol polyproteins and are also packaged into progeny virions as genomic RNA. Nuclear export of these mRNAs requires the existence of a cis acting element, RRE, located in env gene as a specific binding site for Rev. NLS domain of Rev promotes the nuclear
export of viral mRNAs by binding to Crm1, a member of the karyopherin family of nucleo-cytoplasmic-transport factors (142).

**Translation**

Once viral transcripts are exported into the cytoplasm, they are translated to viral proteins essential to the viral life cycle. HIV relies completely on the host cell for protein synthesis, since it does not carry or encode its own translation machinery. The entire translation complex is composed of cellular ribosomes, viral mRNAs, amino-acylated tRNAs, GTP, and several cellular proteins including initiation, elongation, and release factors. Initiation of Eukaryotic mRNA translation is when the small ribosome subunit binds on the 5’cap structure and scans in the 5’ to 3’ direction until it encounters the start codon (AUG) within the Kozak sequence (143). Because HIV RNAs are capped at their 5’ends, it has long been thought that the mechanism for initiation of translation is similar to that used for most cellular mRNAs. However, different from most cellular mRNA, the HIV-1 5’ LTR has extensive RNA secondary structure that typically inhibits ribosome scanning and cap-dependent translation initiation. Sequences in the LTR can function as an internal ribosome entry site (IRES) to drive translation during the G2/M phase of the cell cycle when cap-dependent translation is inhibited (144).

The first gag codon for translation is also the AUG initiator codon. Eukaryotic initiation factor 2B (eIF2B) regulates the binding of the initiator methionyl-tRNA to the ribosome complex, and eukaryotic initiation factor 4E (eIF4E) binds the 5’cap structure of the viral RNA transcript and brings it in contact with the translational machinery (145).
Cellular elongation factors as well as energy derived from GTP hydrolysis are recruited to help the process of translation elongation.

**Ribosomal frameshifting in Gag-pol polyprotein**

Gag-pol polyprotein is produced through a -1 ribosomal frameshifting event at the 3’end of the gag open reading frame (175). Frameshifting is thought to be a form of translational regulation that allows for the correct ratio of Gag to Gag-pol intracellular expression required for efficient viral particle formation.

A programmed -1 ribosomal frameshift requires two elements in the mRNA: (i) a heptanucleotide where the frameshift occurs, called the slippery sequence, with an X XXY YYZ consensus sequence (where the 0 frame is indicated by spaces), and (ii) a secondary structure located downstream of the slippery sequence that stimulates the frameshift. When ribosome bearing two tRNAs, the peptidyl-tRNA in the P site and the aminoacyl-tRNA, in the A site, encounter the slippery sequence, a minority of them shift the reading frame. This recoding event can be described as follows: the two tRNAs whose anticodons interact with the codons of the mRNA in the 0 frame (X XXY YYZ) unpaired from this mRNA, the ribosome shifts backward by 1 nt and the tRNAs re-pair in the -1 frame (XXX YYY) (176). Peptide bond formation then occurs and translation resumes in the new reading frame. The stimulatory secondary structure downstream of the slippery sequence makes the ribosome pause at the slippery sequence (177-179). The RNA fragment encompassing the Gag/Pol frameshift region of HIV-1 has been widely investigated. The HIV-1 Gag/Pol slippery sequence is U UUU UUA, and the frameshift stimulatory signal is suggested to be a two stem helix, made of an upper stem-loop, corresponding to
an 11 bp stem, and a lower stem, formed by pairing the purine-rich sequence upstream of the upper stem loop to the pyrimidine-rich segment downstream. A three purine bulge interrupts these two stems (180).

The frameshift process is crucial to HIV-1 replication as it allows expression of the Gag-pol polyprotein and thus targeting of replicative enzymes to the particle core during assembly. It also sets a precise ratio of Gag: Gag-pol polyprotein, with some 5-10% of translational events yielding Gag-pol. Maintenance of this ratio appears to be essential. Expression of HIV-1 Gag alone, although sufficient for assembly and release of virus-like particles, leads to non-infectious virions lacking indispensable viral enzymes (181). Similarly, expression of the Gag-Pol polyprotein alone is detrimental, resulting in intracellular activation of the HIV-1 protease and inhibition of assembly and budding of virus-like particle (182-184).

Assembly and budding

The process of assembly is mediated by the HIV-1 p55 to generate virus-like particles when expressed in a cell. The infectious particle contains the viral Env, and three structural proteins matrix (MA), capsid (CA), nucleocapsid (NC). Matrix forms the inner shell of the particle, located just under the viral membrane. Capsid forms the conical capsid that encloses the viral genomic RNA. Nucleocapsid interacts with the viral genomic RNA within the capsid.

Scarlata et al proposed the following model for HIV-1 Gag assembly (146). Synthesized on free polysomes in the cytosol, Gag then binds to intracellular vesicles entering the endosomal trafficking system. During this time, Gag interacts with the newly
synthesized viral RNA through the I (interaction) domain on NC, which also promotes Gag-Gag association. Gag oligomerization most likely occurs at the same time with RNA condensation and it is suggested that highly charged free inositol phosphates may assist this condensation process. When Gag enters the endosomal trafficking machinery, the MA region becomes bound to endosomal lipids. The fusion of the endosomal membrane with the plasma membrane is likely to deliver Gag finally to the plasma membrane. The presence of RNA bound to the zinc fingers of NC probably helps to locate Gag in the correct orientation to the membrane surface. The N-terminal region of MA can also contact with the membrane surface and envelope proteins as well. With more Gag-RNA delivered to the membrane, the particle will bud outward from the cell.

Previously it was believed that the location of HIV-1 budding is at the plasma membrane. This appears to be the case for most cells such as CD4+ T-cells, the primary target of HIV-1 infection. However, recent studies show that in macrophages, budding can take place in MVBs (multivesicular bodies). MVBs are membrane bound organelles that serve as endocytic intermediates. Further evidence confirming HIV-1 budding from MVBs in macrophages comes from the presence of MVB markers in the membranes of virus derived from macrophage infection that are not present in virus generated in CD4+ T-cells (147).

**Maturation**

Shortly after virus budding or concurrently, the immature virion will undergo the step of maturation to become an infectious virus particle. The PR cleaves the Gag into structural proteins (MA, CA and NC), and cleaves Gag-pol into viral enzymes (RT, IN
and PR). This process results in the formation of a mature infectious virion with electron-dense and conical core, which is able to invade another target cell.

**HIV-1 Primer Selection**

All retroviruses including HIV-1 utilize a host tRNA as the primer to initiate its reverse transcription, resulting in a double-strand cDNA. The primer tRNA anneals to the PBS (primer binding site) region located at the 5’ end of the viral genomic RNA to prime the synthesis of minus strand cDNA. Different retroviruses selectively prefer different tRNA primers. Most lentiviruses, such as HIV-1, HIV-2, use tRNA^{Lys,3} as a primer, except Visna virus selects tRNA^{lys1,2} as the primer, which is also the primer for human foamy virus and Mason-Pfizer monkey virus. Most avian retroviruses prefer tRNA^{trp} and the majority of murine leukemia viral group choose tRNA^{pro} as a primer. It is already known that PBS sequences are crucial determination for the primer selection, and the U5 region is important as well.

**General properties of tRNA**

Most known tRNAs have approximately 76 nt and may be arranged in the so-called cloverleaf secondary structure. Generally, the tRNA cloverleaf can be divided into five regions: the acceptor stem, D stem-loop, anticodon stem-loop, variable loop, and TWpC stem-loop. The acceptor stem consists of a 5’ terminal phosphate group and a 7 base pair stem. All tRNAs terminate with the ubiquitous sequence, CCA, ending with a 3’ OH group. The 3’ hydroxyl group of the ribose of the terminal adenosine serves as the point of attachment for a unique amino acid. The D stem-loop usually contains the mod-
ified base dihydouridine (D). The anticodon stem-loop, the lower region of the tRNA secondary structure, is composed of a 5 base pair stem ending in a loop containing the anticodon. Above the anticodon stem-loop is the variable loop, which has the greatest variability among tRNAs. There are 15 invariant positions (always the same bases) and 8 semi-invariant positions (only a purine or only a pyrimidine) that are primarily located in the loop regions. It is universal among all tRNAs that the cloverleaf base-pairing tRNA sequence folds into an L-shaped molecule. An L-shaped tRNA can be divided into two main domains: the “top half”, a minihelix motif consisting of the acceptor stem and TψC stem loop, and the “bottom half”, including the anticodon stem-loop, D stem-loop and variable loop. The tRNA is first transcribed by RNA polymerase III (in eukaryotes) as a precursor with 5’ and 3’ extension (leader and trailer sequences, respectively). The two extensional sequences are cleaved or processed by various enzymes for the precursor to have a standard length. Then, many modifications are incorporated into various portions (mainly the core formed by the D-loop and TψC-loop, and the anticodon loop) of this unmodified transcript by numbers of modification enzymes, resulting in a mature tRNA. Finally, aminoacyl-tRNA synthetases specifically recognize the mature tRNA, and ligate the corresponding amino acid onto the 5’CCA terminus (152).

The presence of modified bases in up to 60 different positions is one of the unique features of tRNAs. tRNA modifications have important biological functions, which can be divided into two categories. Modifications located in the tRNA core region (D- and TψC-loop) contribute toward stabilizing the L-shaped tertiary structure. On the other hand, modifications occurring within the anticodon loop have dual functions of precise codon pairing and accurate recognition by the cognate aminoacyl-tRNA synthetases.
Some hypermodified nucleosides strengthen the otherwise relatively weak pairing associations of these bases with the codon, thereby increasing translational fidelity. Conversely, certain methylations block base pairing and hence prevent inappropriate structures from forming. For tRNA used as primer in retroviruses, the modification is considered to be essential for several steps in reverse transcription.

The aminoacylation of tRNAs is one of the most highly conserved processes in nature. It is catalyzed by specific enzymes, termed aminoacyl-tRNA synthetases (aaRSs). The overall aminoacylation reaction is

\[
\text{Amino acid} + \text{tRNA} + \text{ATP} \rightarrow \text{aminoacyl-tRNA} + \text{AMP} + \text{PPi} \quad (153).
\]

At least one aaRS is required for each amino acid. The features of each tRNA to be distinguished by its cognate aminoacyl-tRNA synthetase are called identity elements. These determinants include nucleotides in single-stranded region, base pairs, or structural motifs (154). Aminoacylation of the tRNA molecule is the signal of functionally mature tRNAs. AaRSs vary in their molecular weight, primary sequence and tertiary structure and some aaRSs can form complexes. In mammals, a macromolecular complex is composed of eight different aaRSs including glutaminyl-, isoleucyl-, leucyl-, glutaminyl-, methionyl-, lysyl-, arginyl-, and aspartyl-tRNA synthetase (172). The role of the complex in the aminoacylation of tRNA in vivo is unknown yet.

Interaction between HIV-1 genomic RNA and primer tRNA\textsuperscript{Lys,3}

The mechanism of how host tRNA is captured from cellular environment into the virion remains unknown. Initiation of reverse transcription of the human immunodeficiency virus type 1 RNA genome is a highly regulated process that requires the formation
of a nucleoprotein complex comprising the viral RNA genome, the specific tRNA\textsubscript{Lys,3} primer and the viral reverse transcriptase (RT) enzyme. At least 18 nt at the 3’end of the cellular tRNA\textsubscript{Lys,3} hybridize to a fully complementary sequence within the untranslated leader of the vRNA transcript. The well conserved motif is termed the primer binding site (PBS). RT uses the annealed tRNA primer for synthesis of a cDNA copy and subsequently a double-stranded DNA form of the viral genome. There is accumulating evidence that initiation of reverse transcription is a strictly regulated process in which several additional vRNA-tRNA\textsubscript{Lys,3} interactions play an important role. PBS sequence located at the 5’end of viral genome is complementary to the 18 nucleotides of the 3’end of primer tRNA\textsubscript{Lys,3}. The annealing of 3’end sequence of tRNA\textsubscript{Lys,3} on PBS initiates reverse transcription. Several lines of research suggest that there are additional interactions between the tRNA primer and the viral genome.

The U-rich anticodon of tRNA\textsubscript{Lys,3} was proposed to interact with a single-stranded A-rich motif in the U5 region immediately upstream of the PBS (192-197), which is termed the A-loop. Nuclear magnetic resonance studies of the A-loop region of HIV-1 suggest that the U5 stem-loop is similar to that of a tRNA anticodon (198), making it possible that the A-loop-tRNA\textsubscript{Lys,3} interaction could have similarities to codon-anticodon interactions that occur during translation.

Beerens et al identified an 8-nt motif in the U5 region of HIV-1 RNA (positions +123 to +130) that is critical for tRNA\textsubscript{Lys,3}-mediated initiation of reverse transcription \textit{in vitro} (148). This U5 motif is not important for tRNA annealing but rather for activation of the PBS-bound tRNA primer to initiate reverse transcription and is therefore referred to as the primer activation signal (PAS). Deletion or mutation of the PAS element severely
impairs reverse transcription initiated from the tRNA primer. It was proposed that the PAS interacts with the anti-PAS motif in the TψC arm of tRNA\textsubscript{Lys,3}. It was demonstrated that an in vitro synthesized tRNA\textsubscript{Lys,3} in the absence of base modifications can function as substitute for its natural counterpart in studies of RT binding and reverse transcription; it can also adopt the correct L-shaped structure including that all base pairs and tertiary interactions are formed (149). These facts suggest that synthetic tRNA transcripts contain all necessary sequence and structure requirements for interacting with viral RNA and RT enzyme (150), although modified nucleotides may be important in some other way.

Isel et al (151) constructed a secondary structure model of the HIV-1 RNA/tRNA\textsubscript{Lys,3} complex accounting for enzymatic and chemical probing data. It reveals an unexpectedly complex and compact pseudoknot-like structure in which most of the anticodon loop, the 3' strand of the anticodon stem and the 5' part of the variable loop of tRNA\textsubscript{Lys,3} interact with viral sequences 12 to 39 nucleotides upstream of the PBS. The core of the binary complex is a complex junction formed by two single-stranded sequences of tRNA\textsubscript{Lys,3}, an intramolecular viral helix, and two intermolecular helices formed by the template/primer interaction. Compared to the structure of the free molecules, only the D arm of tRNA\textsubscript{Lys,3} and a small viral stem-loop downstream of the PBS are unaffected in the binary complex.

The role of M\textsuperscript{1}A58 of tRNA\textsubscript{Lys,3} in terminating synthesis of +sss DNA during the HIV life cycle was first mentioned by Gilboa et al (163). This residue is posttranscriptionally modified by the nucleotide specific 1-adenosine methyltransferase, which adds a methyl group to the N1 position of the adenosine base; and it is suggested that this modification abolishes the ability of the A58 residue (now M\textsuperscript{1}A58) to form a stable Watson-
Crick A:T base pair (165,166). Renda et al investigated the role of tRNA$^{\text{Lys,3}}$ residue A58 in the replication cycle of HIV-1 in living cells. They demonstrate that the presence of M$^{1}$A58 is necessary for the appropriate termination of plus-strand strong–stop DNA synthesis and that the absence of M$^{1}$A58 allows RT to read beyond residue 58 during plus-strand strong-stop DNA synthesis and the replacement of M1A58 with U inhibits the replication of HIV-1 in vivo (164). Wu et al revealed multiple termination sites for plus-strand strong-stop DNA synthesis through endogenous RT reactions (165). The first termination site they observed was M$^{1}$A58. In addition, the second termination site was discovered at pseudouridine 55 (ψ55). If M1A58 serves as a stop signal to the +sssDNA synthesis, then if the A58 in tRNA$^{\text{Lys,3}}$ is mutated to U, disrupting the methylation of the nucleotide, the incorporation of c-tRNA attached to the 3’end of plus-strand strong stop DNA is predicted to interfere with completion of the plus strand following second strand transfer, and will result in an abortive replication cycle.

*Determinination of primer tRNA by PBS sequence and A-loop*

Wakefield, et al (155) in our lab changed the HIV-1 proviral PBS sequence to be complementary to the 18 nucleotides of 3’ primer tRNA$^{\text{His}}$. The resulting viruses were discovered to use tRNA$^{\text{His}}$ as primer instead of tRNA$^{\text{Lys,3}}$. However, the viruses reverted back to use tRNA$^{\text{Lys,3}}$ with the PBS sequence reverting back after a short time culture in cell line. When additional mutations were made in the U5 A-loop region to be complementary to the anticodon loop of tRNA$^{\text{His}}$, the resulting virus could maintain the usage of the tRNA$^{\text{His}}$ as primer and PBS sequence remain complementary to tRNA$^{\text{His}}$ over extended *in vitro* culture. This result was further confirmed by studies in continuous T cell
lines of HIV-1 with both PBS and U5 A-loop sequences mutated to be complementary to tRNA^{lys1,2}, tRNA^{Ile}, tRNA^{Pro}, tRNA^{Trp}, tRNA^{Glu}, and tRNA^{Met} (156-160). Viruses with U5 and PBS sequences complimentary to tRNA^{lys1,2} and tRNA^{Met} stably maintained usage of each respective alternate primer after extensive culture, while, viruses with U5 and PBS sequence to be complimentary to tRNA^{Ile}, tRNA^{Trp}, and tRNA^{Pro} reverted back to wild type eventually (156,160).

Yu et al (161) identified and characterized a defective HIV-1 provirus in which the PBS was genetically engineered to be complementary to the 3’ terminal 18 nucleotides of yeast tRNA^{Phe}. This virus was not infectious in tissue culture system unless yeast tRNA was applied in trans and the resulting virus showed a dose-dependence increase in infectivity with the addition of the yeast tRNA. The establishment of this single round complementation system enables us to manipulate both the viral RNA genome as well as the tRNA primer by mutagenesis, which avoids the difficulty of manipulating endogenous levels of tRNAs in eukaryotic cells. Using this complementation system, Yu et al (162) identified that the TψC stem-loop and anticodon stem-loop of yeast tRNA^{phe} are essential in rescuing defective HIV-1 with PBS complementary to yeast tRNA^{phe}, while the D stem-loop structure was not required in the primer selection.

Instead of using yeast tRNA^{Phe} as the provided primer in trans, E.coli tRNA^{Lys,3} is an ideal candidate to examine the role of specific regions or nucleotides of tRNA in primer selection and reverse transcription because a combination of two posttranscriptional anticodon modifications distinguishes the E.coli and mammalian tRNA^{Lys,3} from other tRNA^{Lys} species: wobble position 34 is modified by 5-methoxycarbonylmethyl-2-thiouridine (mcm^5U34) in human tRNA^{lys} and a closely related 5-methylaminomethyl-2-
thiouridine (mmn5's2U34) in *E.coli* tRNA^Lys,3^ . Adenosine-37, 3’adjacent to the anticodon, is modified by 2-methylthio-N6-threonylcarbamoyladenosine (ms2t6A37) in tRNA^Lys,3^ and by t6A37 in the *E.coli* tRNA^Lys,3^. The *E.coli* tRNA^lys^ anticodon stem and the complete anticodon loop are identical to that of mammalian tRNA^Lys,3^ (173). On the other hand, there is enough difference between the mammalian tRNA^Lys,3^ and *E.coli* tRNA^Lys,3^ 3’ terminal 18 nucleotides to allow for specific base-paring between the PBS and tRNA, excluding the endogenous tRNA^Lys,3^ priming. McCulley et al (174) have constructed an HIV-1 provirus with the *E.coli* tRNA^Lys,3^ PBS which requires cotransfection of the plasmid encoding the *E.coli* tRNA^Lys,3^ to generate infectious virus.

*Coupling of primer selection and viral translation*

How the tRNA primer is selected by HIV-1 is not well known. The fact that HIV-1 preference for tRNA^Lys,3^ as the primer can be changed by alteration of the PBS sequence suggests that this virus has evolved a selection process to access the majority of cellular tRNAs; it also suggests that selection of primer must occur at an intracellular site where the virus has access to a variety of tRNAs. The biogenesis of tRNA is a complex and strictly regulated process that requires processing of a tRNA precursor within the nucleus, subsequent transport to the cytoplasm, and incorporation into protein synthesis. It is already clear that the tRNA used as primer for reverse transcription must be de-aminoacylated. However, tRNAs are rarely free in the cytoplasm and are found most frequently associated with cellular proteins (168,169). There are two such subintracellular locations for free tRNA pool: one is nucleus during tRNA biosynthesis and the other one is the cytoplasm during the tRNA translation cycle. By constructing tRNA mutants
that are defective in different steps of tRNA biogenesis and are localized to the nucleus or cytoplasm, Kelly et al demonstrated for the first time that HIV-1 requires the tRNA primer to be transported from the nucleus to the cytoplasm and suggested that the selection of the tRNA for use in reverse transcription occurs at or near the intracellular site of translation (167).

Previous studies from our laboratory had shown that tRNA$^{\text{Met}}$, tRNA$^{\text{His}}$, tRNA$^{\text{Lys}1,2}$ or tRNA$^{\text{Glu}}$ can be stably used as HIV-1 primers when the virus PBS and A-loop were modified to be complementary to responding tRNAs; surprisingly, codons for these amino acids are noted to appear in a region upstream of the frameshift site for Gag-pol polyprotein. Additionally interesting, several HIV-1 with the PBS and A-loop modified to be complementary to tRNA$^{\text{Ile}}$ or tRNA$^{\text{Ser}}$ cannot be stabilized to use these tRNA as primers (155-160). Coincidentally, codons for these amino acids are not found near the Gag-pol frameshift site.

According to the facts mentioned above, a model on HIV-1 acquisition of tRNA primer predicts that the codon usage within HIV Gag-pol might influence the availability of tRNAs for reverse transcription. Approximately 5% of the ribosomes translating HIV-1 Gag were programmed -1 to synthesize the frameshift at the slippery sequence upstream of the gag stop codon to transcribe Gag-pol fusion protein. The -1 slippery sequence is facilitated by the RNA sequence just downstream. The pseudoknot or other secondary structure formed by downstream RNA sequence is likely to result in ribosomal pausing (170,171). At the ribosomal pausing site, the local ribosome density will increase with a concomitant increase of tRNA concentration especially certain uncharged tRNAs at the pausing site. These uncharged tRNA may be incorporated into HIV-
1 virions and used as primer. One consequence of this model is that the codon usage within this frameshift region favors the acquisition of certain tRNAs for primer over others. The codons for tRNA_{Lys,3} and tRNA_{Lys1,2} are the most abundant ones located upstream of frameshift site, which is consistent with the facts that tRNA_{Lys,3} and tRNA_{Lys1,2} are the most enriched tRNA species packaged in the virion and the virus prefers tRNA_{Lys,3} as a primer.

Focus of Dissertation Research

The process of selection and use of tRNA primer for HIV-1 reverse transcription in vivo is so complex that it involves multiple factors. The mechanism underlying this process remains unknown. The roles of viral factors, mainly RT, NC, and the viral genomic RNA, have been studied in most researches in this area, while, little is known about the determinants within the cellular tRNA primer for its selection and use in reverse transcription. The line of research has been hampered by the difficulty in manipulating endogenous tRNA levels in mammalian cells and the tight regulation of the levels of endogenous tRNA as a means to control translation. To circumvent this issue, we have developed a primer complementation system that allows tRNA selected for reverse transcription to be supplied in trans. This system relies on the use of \textit{E.coli} tRNA_{Lys,3}, which maintains many of the features of the mammalian tRNA_{Lys,3} but has enough difference in 3’end 18 nucleotides to distinguish it from cellular tRNA_{Lys,3}.

We have engineered HIV to contain a PBS complementary to the 3’end 18 nucleotides of \textit{E.coli} tRNA_{Lys,3}. The infectivity of HIV in the system depends on providing \textit{E.coli} tRNA_{Lys,3} in trans. In the current research, we explore the effects of several el-
ments within the TΨC stem-loop of *E.coli* tRNA$^{\text{Lys,3}}$ on the infectivity of HIV which is forced to specifically use *E.coli* tRNA$^{\text{Lys,3}}$ as a primer. All experiments were conducted by Wanfeng Yu, the author of current thesis except that Anna McCulley constructed the proviral genome, Ec PBS NL4-3 and part of the northern blot. In the second manuscript, we have used this complementation system to further explain the link between viral translation and primer selection. A recent study has shown that alteration of the five lysine codons to be specific for tRNA$^{\text{Lys1,2}}$ prior to the Gag-pol junction enhanced the replication of HIV-1 forced to use tRNA$^{\text{Lys1,2}}$. In the second manuscript, the effect of altering the lysine codons in Gag to be specific for tRNA$^{\text{Lys,3}}$ or tRNA$^{\text{Lys1,2}}$ has on complementation. The results of these studies further support the link between viral translation of the Gag-pol protein and primer selection. Wanfeng Yu conducted all the complementation experiments and all the mutations in the *E.coli* tRNA$^{\text{Lys,3}}$ genes. Fragments containing F3, F5 mutations were constructed by Matthew Palmer in our lab, and fragment with BH mutations were provided by Richard Kirkman in our lab.
MUTATIONS IN THE TΨC LOOP OF *E. coli* tRNA\textsuperscript{Lys,3} HAVE VARIED EFFECTS ON *IN TRANS* COMPLEMENTATION OF HIV-1 REPLICATION

by

WANFENG YU, ANNA MCCULLEY AND CASEY D. MORROW

Accepted by Virology Journal

Format adapted for dissertation
Abstract

Background

Human immunodeficiency virus (HIV-1) exclusively selects and utilizes tRNA\textsubscript{Lys,3} as the primer for initiation of reverse transcription. Several elements within the TΨC stem loop of tRNA\textsubscript{Lys,3} are postulated to be important for selection and use in reverse transcription. The post-transcriptional modification at nucleotide 58 could play a role during plus-strand synthesis to stop reverse transcriptase from re-copying the tRNA primer. Nucleotides 53 and 54 within the TΨC stem loop of the tRNA have been shown to be important to form the complex between tRNA and the HIV-1 viral genome during initiation of reverse transcription.

Results

To further delineate the features of the TΨC stem loop of tRNA\textsubscript{Lys,3} in reverse transcription, we have developed a complementation system in which \textit{E. coli} tRNA\textsubscript{Lys,3} is provided \textit{in trans} to an HIV-1 genome in which the PBS is complementary to this tRNA. Successful selection and use of \textit{E. coli} tRNA\textsubscript{Lys,3} results in the production of infectious virus. We have used this single round infectious system to ascertain the effects that different mutants in the TΨC stem loop of tRNA\textsubscript{Lys,3} have on complementation. Mutants were designed within the TΨC loop (nucleotide 58) and within the stem and loop of the TΨC loop (nucleotides 53 and 54). Analysis of the expression of \textit{E. coli} tRNA\textsubscript{Lys,3} mutants revealed differences in the capacity for aminoacylation, which is an indication of intracellular stability of the tRNA. Alteration of nucleotide 58 from A to U (A58U), T54G and TG5453CC all resulted in tRNA\textsubscript{Lys,3} that was aminoacylated when expressed in cells, while a T54C mutation resulted in a tRNA\textsubscript{Lys,3} that was not aminoacylated. Both
the A58U and T54G mutated tRNA$^{\text{Lys,3}}$ complemented HIV-1 replication similar to wild type $E.\ coli$ tRNA$^{\text{Lys,3}}$. In contrast, the TG5453CC tRNA$^{\text{Lys,3}}$ mutant did not complement replication.

**Conclusions**

The results demonstrate that post-transcriptional modification of nucleotide 58 in tRNA$^{\text{Lys,3}}$ is not essential for HIV-1 reverse transcription. In contrast, nucleotides 53 and 54 of tRNA$^{\text{Lys,3}}$ are important for aminoacylation and selection and use of the tRNA$^{\text{Lys,3}}$ in reverse transcription.
Background

The major steps in reverse transcription of retroviral genome have been known for some time [1]. The initiation of reverse transcription occurs at the 5’ end of the viral genome at a site designated as the primer-binding site (PBS) [1]. The PBS is an 18-nucleotide region that is complementary to the 3’ terminal 18-nucleotides of the tRNA primer used for initiation [1-3]. The reverse transcriptase extends the bound tRNA primer from the PBS resulting in the synthesis of minus strong stop DNA [4]. The reverse transcriptase then translocates to the 3’ end of the viral RNA genome and proceeds to generate a complete minus-strand DNA copy of the viral RNA genome. The RNaseH activity of the viral encoded reverse transcriptase degrades the RNA copy of the viral RNA genome. Incomplete processing of the RNA by the RNaseH activity generates RNA primers for plus-strand DNA synthesis [4]. During plus-strand synthesis, the reverse transcriptase copies the tRNA primer that is attached to the minus-strand DNA to generate a plus-strand copy of the PBS. Complementation between the plus- and minus-strand PBS facilitates the completion of the viral genome, designated as the provirus.

The vast majority of the studies that have analyzed the mechanistic events of reverse transcription have utilized in vitro systems comprised of tRNA, reverse transcriptase, nuclear capsid and synthetic viral RNA/DNA templates. Previous studies have found that the tRNA$^{\text{Lys,3}}$ and the HIV-1 genome form a complex RNA structure for initiation of reverse transcription. As a consequence of this tRNA:RNA genome interaction, the tRNA$^{\text{Lys,3}}$ structure is disrupted and new intramolecular bonds are formed. One important new RNA:RNA interaction is between nucleotides 53 and 54 and the first two nucleotides of tRNA$^{\text{Lys,3}}$ [5, 6].
While *in vitro* studies have been informative in understanding the aspects of reverse transcription, they do not completely recapitulate all of the events in replication of the viral RNA genome. Our laboratory has approached this problem by generating HIV-1 proviruses that require the addition of exogenous tRNA for infectivity. In previous studies, we utilized an HIV-1 proviral genome in which the PBS had been mutated to be complementary to yeast tRNA$^{\text{Phe}}$\[7-10\]. We found that the replication of this genome could be complemented if yeast tRNA$^{\text{Phe}}$ was supplied *in trans*. *In vitro* systems with synthetic tRNA/viral templates have been used to characterize many of the features of reverse transcription \[11\]. An important question that has been addressed using these systems is the role of modified tRNA bases that might play a role in stopping the reverse transcriptase during the plus-strand DNA synthesis to prevent complete copying of the tRNA primer. Since the completion of the proviral genome is facilitated by complementarity between the minus- and plus-strand DNA copies of the PBS, additional sequences in the plus-strand copy of the PBS as a result of copying of the tRNA primer would compromise the completion of the proviral genome. Previous studies have suggested that the methylated adenosine residue at position 58 (A58) of the tRNA could be a stop signal for the reverse transcriptase \[12-14\]. Support for this result comes from studies by Renda *et al.* who found that tRNA$^{\text{Lys},3}$ engineered to not be methylated at A58 residue conferred a level of resistance to cells expressing this tRNA \[13, 14\]. Additional studies though have suggested that the methylated A58 residue is not the sole stop determinant in plus-strand DNA synthesis \[12\].

In a recent study, we have engineered a complementation system which utilizes an HIV-1 proviral genome in which the PBS has been altered to be complementary to the
3’ terminal 18-nucleotides of \textit{E. coli} tRNA$^{\text{Lys,3}}$ [15]. This tRNA maintains many of the unique transcriptional modifications found in mammalian tRNA$^{\text{Lys,3}}$, and when expressed in mammalian cells, has been shown to be aminoacylated indicating that it is fully functional [15]. Thus, this system provides an excellent opportunity to directly address the role of the modified nucleotides and tRNA structure in HIV-1 reverse transcription.

In the current study, we have found that \textit{E. coli} tRNA$^{\text{Lys,3}}$ with mutations at nucleotides 58, 54 and 53 in the TΨC loop region have varied effects in the production of infectious HIV-1. The results of our studies demonstrate that features in the TΨC loop of tRNA$^{\text{Lys3}}$ are important for the selection and use of the tRNA as a primer for HIV-1 reverse transcription.
Materials and Methods

Tissue culture.

293T cells, JC53-BL cells, and HeLa H1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (Gibco/BRL, Gaithersburg, MD). All cells were cultured in 37°C incubator supplied with 5% CO₂.

Plasmid construction.

The LS9 plasmid containing *E. coli* tRNA<sup>Lys</sup> gene and LS9 plasmid containing mammalian tRNA<sup>Lys</sup> gene were constructed as previously described [15]. The *E. coli* tRNA<sup>Lys</sup> gene in LS9 is located downstream of the human U6snRNA promoter. The A58 in *E. coli* tRNA<sup>Lys</sup> was mutated to T using the QuickChange Side-Directed Mutagenesis (Stratagene, La Jolla, CA) with Ec A58U primers: (Ec A58U forward) 5’GG TCGTCAGGACATGAACCTGCGAC3’, (Ec A58U reverse) 5’GTCGCAGGTTCATGTCTGCACGACC3’. T54 in *E. coli* tRNA<sup>Lys</sup> was substituted to G with Ec T54G primers: (Ec T54G forward) 5’CAATTGCTGCGAGGACTTGCCTGCACGACCC3’, (Ec T54G reverse) 5’GGGTCGTGCAGGACTTGAGGCTGCGACCAATTG3’. T54 in *E. coli* tRNA<sup>Lys</sup> was also substituted to C with Ec T54C primers: (Ec T54C forward) 5’CAATTGCTGCGAGGCTTGCCTGCACGACCC3’, (Ec T54C reverse) 5’GGGTCGTGCTGCGACCAATTG3’. TG5453 together were substituted to CC with EcTG/CC primers: (Ec TG/CC forward) 5’GACTTTTAATCAATTGCTGCGACCAATTG3’, (Ec TG/CC reverse) 5’GGGTCGTGCTGCGACCAATTG3’. All mutations were verified by DNA sequencing.
The PBS of the proviral HIV-1 genome (NL4-3) was substituted for a PBS complementary to the 3’ terminal 18-nucleotides of E. coli tRNA\textsuperscript{Lys,3} by mutagenesis as described in [15]. A PBS shuttle vector with the substituted PBS was digested using the restriction enzymes BssHII and HpaI in order to release the fragment containing the E. coli tRNA\textsuperscript{Lys,3} PBS region (an 868-bp fragment). The isolated fragment was ligated into the pNL4-3, which was also digested using the enzymes BssHII and HpaI. Resulting HIV-1 proviral mutant was labeled NL4-EcoLys3. Final mutants were verified by DNA sequencing.

**DNA transfections.**

Co-transfections were performed according to the protocol for the Fugene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, 293T cells were seeded at a concentration of 2 x 10\textsuperscript{5} cells per well in 6-well plates. 500 ng of NL4-EcoLys3 and 100 ng, 250 ng, 500 ng or 1000 ng LS9 plasmids encoding E. coli tRNA\textsuperscript{Lys,3} (wild type or mutations) and 3 μl Fugene reagents were added to 100 ul of DMEM. These mixtures were incubated a room temperature for approximately 45 minutes then added drop-wise to 6-well plates. The cells were supplied with fresh media 24 hours post transfection. Supernatants were collected approximately 48 hours post transfection, centrifuged at 3,000g, and used in JC53-BL assay to determine luciferase activity, which has been determined to correlate to units of infectious virus that is being tested. Supernatants were also assayed for HIV-1 p24 antigen (Beckman Coulter, Miami, FL).
Analysis of virus infectivity.

Serially diluted supernatants collected from co-transfections were used to infect JC53-BL cells to determine viral infectivity. JC53-BL cells were seeded 24 hours pre-infection. Infected cells were incubated for 2 hours in 37°C incubator supplemented with 5% CO₂. After 2 hours, DMEM with 10% FBS was added to each well and the cells were incubated for additional 48 hours. To determine luciferase activity, cells were lysed using M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) and approximated 20 µL of each lysed sample were transferred to a microplate. Reporter Lysis Buffer (Promega, Madison, WI) was added to each sample in the microplate and the light intensity was measured using a Tropix TR717 Microplate Luminometer (Applied Biosystems, Foster City, CA). Uninfected cells in wells represented background luciferase activity which was subtracted from all other samples. Relative Light Units (RLu) per mL were calculated by dividing the luciferase values by their corresponding dilutions. The total amount of virus was determined by the p24 ELISA. The amount of infectious virus was determined as RLU per nanogram p24.

Analysis of *E. coli* tRNA^{Lys,3} expressed in mammalian cells.

*E. coli* tRNA^{Lys,3} plasmids were transfected into 293T cells, and total RNA was extracted 48 hours post transfection under acidic conditions to maintain the amino acid-tRNA bond [15]. One-half of the RNA sample was treated with high pH (pH 9) to serve as a de-aminoacylated control. The samples were separated in an acidic polyacrylimide gel as previously described [15]. Northern blot was carried out using NorthernMax-Gly kit (Ambion) using previously described conditions for isolation of total RNA [15]. The probe for *E. coli* tRNA^{Lys,3} 5’GGTCGTGCAGGATTCACTGCGACCAATTGATT
AAAAGTCAACTGCTCTACCAACTGAGCTAACGAC3’ was phosphorylated using the ready to-go kit (Amersham) with [γ-32P-ATP]. Hybridization was carried out under standard conditions. The blots were exposed to X-ray film which was developed using an SRX-101A developer (Konica, Wayne, NJ).

**PCR and DNA sequence analysis of the PBS regions from integrated proviruses.**

High molecular weight DNA (HMW) was collected using Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The region encompassing the PBS was amplified by PCR using the following primers: (forward) 5’TAGACCAGATCTGAGCCTGGGAGCTC3’ and (reverse) 5’CTCCTTCTAGCCTCGCTAGTC3’. Following PCR, the products were run on 1% agarose gel and gel extracted with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and used for DNA sequencing.
Results

Construction of *E. coli* tRNA$^{\text{Lys},3}$ mutants and HIV-1 proviral genomes.

In a recent study, we have described a complementation system which relies on the addition of *E. coli* tRNA$^{\text{Lys},3}$ *in trans* to complement an HIV-1 proviral genome in which the PBS was altered to be complementary to the 3’ terminal 18-nucleotides of this tRNA [15]. *E. coli* tRNA$^{\text{Lys},3}$ shares many features with mammalian tRNA$^{\text{Lys},3}$. In a previous study, we demonstrated that transfection of the proviral genome with a PBS to *E. coli* tRNA$^{\text{Lys},3}$ into mammalian cells required complementation by *E. coli* tRNA$^{\text{Lys},3}$ cDNA in order to produce infectious virus [15]. Titration of increasing amounts of *E. coli* tRNA$^{\text{Lys},3}$ plasmid resulted in corresponding increase in levels of infectious virus.

In the current study, we have constructed mutants in the *E. coli* tRNA$^{\text{Lys}}$ gene in which the A58 was mutated to T (A58U) (Figure 1A). To characterize the effect of this mutation we ascertained whether the tRNA could undergo aminoacylation following transfection of tRNA plasmid into mammalian cells. The capacity of the tRNA to undergo aminoacylation is indicative of proper identity elements, three-dimensional folding of the tRNA, and, most probably, inclusion into the translational machinery (Figure 1B). Following transfection of the wild type *E. coli* tRNA$^{\text{Lys},3}$ and *E. coli* tRNA$^{\text{Lys},3}$ A58U into mammalian cells, we analyzed these cells for the presence of aminoacylated *E. coli* tRNA$^{\text{Lys},3}$. The wild type and mutants were expressed in mammalian cells following transfection and aminoacylation (Figure 1B). Thus, the lack of post-transcriptional A58 modification did not influence the capacity of this tRNA to undergo expression, transport from the nucleus to the cytoplasm, aminoacylation, and presumably inclusion into the translational machinery.
Next, we wanted to determine whether the mutant *E. coli* tRNA\textsuperscript{Lys,3} A58U could complement the HIV-1 proviral genome in which the PBS was altered to be complementary to the 3’ terminal 18-nucleotides of this tRNA (Figure 1C). For these studies, we utilized increasing amounts of plasmids encoding the wild type and mutant *E. coli* tRNA\textsuperscript{Lys,3} in the transfection. The amount of virus released from the transfections was determined using the JC53-BL assay. Complementation of the proviral genome was demonstrated following transfection of the wild type *E. coli* tRNA\textsuperscript{Lys,3}. The levels of infectious virus increased reaching a plateau at approximately 500 ng of plasmid encoding the tRNAs. Importantly, we also found a similar level of complementation following transfection of *E. coli* tRNA\textsuperscript{Lys,3} A58U, confirmed by production of infectious virus. In this case, we found no clear difference between the wild type and mutant tRNA for the capacity to complement the HIV-1 proviral genome. Analysis of the PBS from the integrated proviruses after infection revealed that all were complementary to *E. coli* tRNA\textsuperscript{Lys,3} (data not shown). We found no evidence for additional tRNA sequences (after nucleotide A58) as would be expected if the reverse transcriptase had not stopped during copy of the tRNA during plus-strand synthesis. Collectively, the results of these studies show that the A58U mutation in tRNA\textsuperscript{Lys,3} does not impact on the capacity of this tRNA to be selected and used in HIV-1 replication.

**Characterization of mutations within the TΨC stem loop of *E. coli* tRNA\textsuperscript{Lys,3}**.

Nucleotides within the TΨC stem loop were modeled to interact with HIV genome in forming the initiation complex between tRNA and viral RNA (Figure 3) [5, 6]. A second interaction has also been found involving the tRNA and nucleotides within the stem of the TΨC stem loop that has also been termed the primer activation signal (PAS)
To begin to address the importance of the ΨC stem loop, we made additional mutations in *E. coli* tRNA\textsubscript{Lys,3}. The first mutation targeted nucleotide 54 which is a T (Figure 2A). Two mutations were made in which the thymidine at nucleotide 54 was changed to a G or C (T54G or T54C, respectively). We also made a third mutation in which the TG at nucleotides 54 and 53 were altered to Cs (TG5453CC) (Figure 2B). Each of these mutations were postulated to affect elements of the initiation complex between the tRNA and HIV viral genome.

We first characterized the effects of these mutation on *E. coli* tRNA\textsubscript{Lys,3}. We determined whether the mutant tRNAs would be expressed and aminoacylated. The capacity of the tRNA to be aminoacylated correlates with the stability of this tRNA within the cell. Analysis of the aminoacylation status reveals that both T54G and TG5453CC *E. coli* tRNA\textsubscript{Lys,3} mutants were predominately aminoacylated within the cell, indicating their intracellular stability. In contrast, the T54C mutation in *E. coli* tRNA\textsubscript{Lys,3} was poorly aminoacylated and consequently expressed at a lower level than the other mutants (data not shown).

We next analyzed the capacity of these *E. coli* tRNA\textsubscript{Lys,3} mutants to complement the replication of the HIV viral genome with the PBS complementary to *E. coli* tRNA\textsubscript{Lys,3} (Figure 2C). Fixed amount of proviral plasmid and increasing amounts of plasmids encoding wild type or mutant *E. coli* tRNA\textsubscript{Lys,3} were co-transfected into mammalian cells. The activity of the resultant virus was determined using the JC53-BL assay. The mutant T54G *E. coli* tRNA\textsubscript{Lys,3} readily complemented the infectivity of the mutant provirus at levels similar to that of the wild type tRNA. It was possible that the T54G *E. coli* tRNA\textsubscript{Lys,3} was slightly more efficient in complementation as evidenced by increased
levels at lower amounts of plasmid co-transfected with the proviral genome. However, the peak levels of complementation were similar between T54G E. coli tRNA^{Lys,3} and the wild type E. coli tRNA^{Lys,3}. Not surprisingly, the mutant T54C E. coli tRNA^{Lys,3} did not complement the infectivity (data not shown). Most probably this was due to poor levels of expression as a result of inability to become aminoacylated. Surprisingly, the TG5453CC E. coli tRNA^{Lys,3} did not complement the mutant proviral genome, even though this tRNA was expressed and aminoacylated in cells. This lack of complementation was observed throughout the entire range of the plasmids used in the titration. Thus, the results of these studies demonstrate that mutations within the TΨC loop can impact the stability of the tRNA (T54C) as well as the capacity of this tRNA to be selected and used in HIV-1 reverse transcription. The results support a role for nucleotides 54 and 53 within the TΨC stem loop in the use of tRNA^{Lys,3} in reverse transcription.
Discussion

Previous studies have examined the role that post-transcriptional modification of the tRNA^{Lys,3} have on HIV-1 reverse transcription [12-14, 17]. In one study, an *in vitro* system was established which recapitulates minus-strand strong stop synthesis and the plus-strand DNA synthesis. The results from these studies establish that the modified nucleotide A58 of the natural tRNA^{Lys,3} was only partially effective as a stop signal [12]. The reverse transcriptase in some instances could transcribe as far as the hypermodified adenosine at A37 in the anticodon loop [17]. Based on the results of these studies, the authors concluded that the modified nucleoside at A58, which is present in all tRNA^{Lys,3} molecules, appears to be important for both the efficacy and fidelity of plus-strand DNA transfer. Renda et al., extended this work and constructed cell lines or derived MuLV based vectors, to express the A58U tRNA^{Lys,3} [13, 14]. Analysis of the replication of HIV-1 in these cells revealed that it was slower than that observed for replication of the virus in cells which did not express the mutated tRNA^{Lys,3}, although the virus did eventually grow in these cells. Analysis of the resultant virus revealed that it had not undergone alteration in the PBS region. However, the inhibition of HIV-1 replication varied in individual cell clones, with some cell clones showing no inhibition. In addition, the levels of the mutated tRNA^{Lys,3} were not determined in the individual cell lines, making it difficult to evaluate how the levels of mutant tRNA^{Lys,3} effect viral replication and cellular metabolism. The results of these experiments suggested that mutations in A58 would have been expected to affect the capacity to produce infectious HIV-1. To further explore the potential of the A58 mutation to inhibit HIV-1 replication, we decided to determine whether the mutant tRNA would complement HIV-1 replication when
provided *in trans*. For our studies, we engineered the HIV-1 proviral genome so that the PBS would be complementary to the 3’ terminal nucleotides of *E. coli* tRNA^Lys,3^. In a recent study, we have shown that *E. coli* tRNA^Lys,3^, when provided *in trans* to this HIV-1 proviral genome, results in production of infectious virus [15]. If the A58 post-transcriptional modification was important for selection and use as the primer, we anticipated that co-transfection with the mutant proviral genome would not result in production of infectious virus. However, we demonstrated that the A58U mutant complemented the infectivity of the HIV-1 proviral genome at levels similar to that observed with the wild type *E. coli* tRNA^Lys,3^. At present, we cannot resolve the differences from our study with those of Renda et al [13, 14]. The results of our study are consistent with the possibility that additional features of the tRNA^Lys,3^ are more important than the modified bases for the termination of plus-strand synthesis [12]. Since our *in vivo* complementation system has all of the appropriate viral and host cell proteins available for the process of reverse transcription, which is not the case entirely for the *in vitro* system, it is possible that other protein and RNA elements can compensate for the lack of modified bases. Indeed, the *in vivo* complementation system recapitulates both the selection process as well as the events in reverse transcription. It is also likely that the three dimensional structure of the tRNA impacts on plus-strand DNA stop [12]. Previous studies have found a complex refolding of the tRNA that occurs during initiation of the minus-strand strong stop DNA synthesis [5, 6]. As a consequence, new intramolecular bonds are established in the tRNA. The new tRNA structure could be a major determinant in the effective plus-strand strong stop. The viral nucleocapsid could facilitate the maintenance of the new tRNA structure. The intracellular *in trans*
complementation system used in the study then recapitulates the appropriate nucleocapsid-RNA interactions, which could explain the production of infectious virus using the tRNA\textsuperscript{Lys,3} mutants. Just how the modified bases affect the new three-dimensional RNA structure is unknown and will require additional studies.

To further explore the role of nucleotides in the tRNA\textsuperscript{Lys,3} TΨC stem loop during reverse transcription, we made additional mutations at nucleotides 54 and 53. The T54G mutation in \textit{E. coli} tRNA\textsuperscript{Lys,3} did not affect the capacity of this tRNA to be aminoacylated or to be used in HIV reverse transcription. In contrast, mutation T54C resulted in lack of aminoacylation and consequently this \textit{E. coli} tRNA\textsuperscript{Lys,3} mutant did not complement HIV replication. Analysis of the expression level for T54C \textit{E. coli} tRNA\textsuperscript{Lys,3} revealed a decrease, upon comparison to the T54G \textit{E. coli} tRNA\textsuperscript{Lys,3}, and lack of aminoacylation by the lysyl-tRNA synthetase. The lower levels of expression found for tRNAs that are unable to undergo aminoacylation is consistent with previous studies that have shown unaminoacylated tRNA instability within cells [18]. An interesting result was obtained with the double mutation TG5453CC. In this case, the TG5453CC \textit{E. coli} tRNA\textsuperscript{Lys,3} was aminoacylated and generally was produced at levels similar to that of the wild type tRNA. However, this tRNA did not complement the HIV-1 proviral genome at all tRNA plasmid concentrations tested. At present, we believe that the TG5453CC mutation in tRNA\textsuperscript{Lys,3} precludes or retards the tRNA from forming an initiation complex with the HIV-1 RNA genome. Previous studies have established that nucleotides with the TΨC stem loop within the \textit{E. coli} tRNA\textsuperscript{Lys,3} could be involved in two potential steps during initiation of HIV-1 reverse transcription. In the first, this region is postulated to form an intramolecular bond with the 5’ end of tRNA; this RNA:RNA interaction in tRNA\textsuperscript{Lys,3} is
postulated to help form the structure for the initiation of HIV reverse transcription [5, 6] (Figure 3). However, the T54G and TG54CC mutations could be predicted to have different effects on the intramolecular tRNA\textsubscript{Lys,3} interactions formed during initiation. The T54G mutation would be predicted to destabilize the interaction to an unfavorable base pair between nucleotide 1 and 54 (G:G). In contrast, the TG5453CC mutation was expected to promote this interaction through G:C base pairs. Thus, it was surprising that the T54G mutation in \textit{E. coli} tRNA\textsubscript{Lys,3} still allowed complementation, while the TG5453CC mutated tRNA\textsubscript{Lys,3} did not complement. It is possible that sufficient base pair interactions still existed to form the initiation complex, but other tRNA:HIV-1 genome interactions were compromised as a result of the TG5453CC mutation (Figure 3). A second function for this sequence has been found to interact with the HIV-1 genome within the U5 region (PAS) [16]. The TG5453CC mutation would have been predicted to only partially disrupt this interaction, but it could have been sufficient to inhibit initiation. Additional studies will be required to further delineate the critical intra and intermolecular interaction between the T\PsiC region of tRNA\textsubscript{Lys,3} and the viral genome for initiation of reverse transcription.
Conclusion

In the current study, we have investigated the contribution that post-transcriptional modification of tRNA$^{\text{Lys,3}}$ at nucleotide A58 and nucleotides within the TΨC stem loop (54 and 53) have on the capacity of this tRNA$^{\text{Lys,3}}$ to be selected and used as the primer for HIV-1 reverse transcription. We found that these mutations, with the exception of T54C, did not affect the expression of these tRNAs and the capacity to undergo aminoacylation, and presumably, entrance into the translational processes of the cell. The capacity of these mutated tRNAs to complement HIV-1 replication, when provided intracellularly in trans was determined by co-transfection with a proviral plasmid in which the PBS was mutated to be complementary to E. coli tRNA$^{\text{Lys,3}}$. From the results of our studies, we conclude that the post-transcriptional modification at nucleotide 58 is not essential for function of tRNA$^{\text{Lys,3}}$ in HIV-1 replication. Nucleotides within the TΨC loop are also important for tRNA:HIV-1 genome RNA interactions in initiation of reverse transcription. We found that one mutant, TG5453CC, could undergo aminoacylation, indicating the tRNA structure was intact, but it did not complement HIV-1 replication even though this mutant would be predicted to have favorable interactions for the initiation complex between tRNA and HIV-1 RNA. A second mutant, T54G, which would be predicted to not favor the tRNA:HIV-1 genome RNA interaction in the initiation complex was fully functional for complementation. From these results, we conclude that a complex interaction between the HIV-1 viral genome in tRNA$^{\text{Lys,3}}$ probably involves multiple, complex RNA:RNA interactions during primer selection and reverse transcription. The absolute base pair complementarity are not necessary for these
interactions, consistent with the idea the RNA structures are dynamic during HIV-1 reverse transcription.
Acknowledgements

We thank members of the Morrow Laboratory for helpful suggestions. Adrienne Ellis is thanked for preparation of the manuscript. AM was supported by training grant (AI07493). The DNA sequencing was carried out by the UAB CFAR DNA Sequencing Core (AI 27767). CDM acknowledges helpful suggestions from MAR. This research was supported by a grant from the NIH (AI34749).
References


Figure 1: Complementation of HIV-1 infectivity with *E. coli* tRNA<sup>Lys<sub>3</sub></sup> A58U mutant.

**Panel A.** Diagram of *E. coli* tRNA<sup>Lys<sub>3</sub></sup> A58U mutant. The base change A58U is indicated. Boldface nucleotides indicate the 3’ 18-nucleotides complementary to the PBS of HIV-1 (*E. coli* tRNA<sup>Lys<sub>3</sub></sup>).

**Panel B.** Expression and aminoacylation of *E. coli* tRNA<sup>Lys<sub>3</sub></sup> and *E. coli* tRNA<sup>Lys<sub>3</sub></sup> A58U in 293T cells following transfection. Total RNA was isolated under acidic conditions to stabilize the amino acid tRNA bond. Approximately one-half was treated with high pH as to break the amino acid-tRNA bond (deAA). Samples were run on an acid polyacrylamide gel and blotted into nitrocellulose. All samples were analyzed with a probe specific for tRNA<sup>Lys<sub>3</sub></sup> [15]. The migration of aminoacylated tRNA (Lane 2) and deacylated controls (Lane 1) are denoted as AA and deAA, respectively. NT is RNA from non-transfected 293T cells.

**Panel C.** Infectivity of NL4-EcoLys3 complemented by *E. coli* tRNA<sup>Lys<sub>3</sub></sup> or *E. coli* tRNA<sup>Lys<sub>3</sub></sup> A58U. 0.5μg NL4-EcoLys3 was co-transfected with 0.1, 0.25, 0.5, 1.0 μg plasmids encoding *E. coli* tRNA<sup>Lys<sub>3</sub></sup> or mutant into 293T cells. Virus was collected 48 hours post transfection. The amounts of infectious virus produced from transfection were determined using the JC53-BL bioassay which measures luciferase activity [19]. The infectivity is determined by the amount of luciferase is divided by the amount of virus as determined by p24 ELISA, to give RLU per nanogram. Values are the average (+SD) from three assays.
Figure 2: Complementation of NL4-EcoLys3 with *E. coli* tRNA<sup>1253</sup> mutants.

**Panel A.** Diagram of *E. coli* tRNA<sup>1253</sup> T54G and T54C. The mutated nucleotide is indicated by a circle.

**Panel B.** Nucleotide diagram *E. coli* tRNA<sup>1253</sup> TG54GG. The mutated nucleotides are indicated by circles.

**Panel C.** Infectivity of NL4-EcoLys3 complemented by *E. coli* tRNA<sup>1253</sup>, *E. coli* tRNA<sup>1253</sup> T54G, and *E. coli* tRNA<sup>1253</sup> TG5453CC mutants. 293T cells were co-transfected with 0.5 µg of proviral plasmid and with tRNA plasmids that were titrated in at the indicated quantities. Infectivity, for complementation of plasmid NL4-EcoLys3 with *E. coli* tRNA<sup>1253</sup> T54G is specified by open circle; NL4-EcoLys3 with wild type *E. coli* tRNA<sup>1253</sup> is specified by closed circle; NL4-EcoLys3 with *E. coli* tRNA<sup>1253</sup> TG5453CC is specified by open square. The infectivity is determined by the amount of luciferase determined by JC53-BL assay divided by amount of virus (p24 ELISA) to obtain RLu per nanogram. Values are the average (+SD) from three assays.
Figure 3: Potential RNA secondary structure of the complex formed by NL4-EcoLys3 viral RNA and E. coli tRNA\(^{\text{Lys3}}\). The tRNA:RNA structure was adapted from that described in previous studies [5, 6]. The mutations at nucleotides 54 and 53 of E. coli tRNA\(^{\text{Lys1,2}}\) are boxed. The T54G mutation would be predicted to destabilize the interaction (G:G), while the TG5453CC mutation should stabilize by favorable G:C interaction.
INFECTIVITY OF HIV-1 THAT REQUIRES *E. coli* tRNA$^{\text{Lys},3}$ SUPPLIED *IN TRANS* IS IMPACTED BY CHANGING tRNA$^{\text{Lys},1,2,3}$ CODON ISOACCEPTOR USE IN GAG

by

WANFENG YU, ANNA MCCULLEY, MATTHEW PALMER, RICHARD KIRKMAN AND CASEY D. MORROW

Manuscript prepared for Virology Journal

Format adapted for dissertation
Abstract

Human immunodeficiency virus (HIV-1) preferentially selects tRNA$^{\text{Lys},3}$ as the primer for reverse transcription. Our recent study has suggested a link between the process of primer selection and lysine codon in the junction prior to this Gag-pol polyprotein during viral replication. To further explore this relationship, proviral genomes were created in which the five codons specific for lysine prior to the Gag-pol frameshift region were modified to be specific for tRNA$^{\text{Lys},3}$ or tRNA$^{\text{Lys},1,2}$. Since it is difficult to manipulate the endogenous levels of mammalian tRNAs, previous studies from this laboratory a complementation system in which E. coli tRNA$_{\text{Lys},3}$ provided in trans to an HIV-1 in which the PBS was modified to be complementary to the 3’ terminal 18-nucleotides of this tRNA. Transfection of proviral genomes with the altered codons produced similar amounts of the virus as the wild type as determined by p24 antigen capture ELISA. In contrast, greater amounts of infectious virus were produced following co-transfection of proviral genome in which all five lysine codons were specific for tRNA$_{\text{Lys},3}$ compared to the wild type or viral genomes in which the five codons were specific for tRNA$_{\text{Lys},1,2}$. To extend this work, viral genomes were created in which all of the lysine codons prior to the five preceding the Gag-pol frameshift site were modified to be specific for tRNA$_{\text{Lys},1,2}$. Analysis of the production of infectious virus from transfection of these proviral genomes revealed that the levels were less regardless of whether the five codons preceding the Gag-pol frameshift were specific for tRNA$_{\text{Lys},3}$ or tRNA$_{\text{Lys},1,2}$. The results of these studies then demonstrate that alteration of the lysine codon frequencies within the Gag protein of HIV-1 can have profound impact on the production of infectious virus. The results of these studies then further support a link
between viral translation and the process by which HIV-1 selects the tRNA primer used for reverse transcription.
Introduction

The hallmark of HIV replication is the process by which the viral RNA genome is converted to a DNA form prior to integration into the host cell chromosome (Varmus, 1988; Varmus, 1982). This process, termed reverse transcription, is catalyzed by a viral encoded protein reverse transcriptase (Baltimore, 1970; Temin and Mizutani, 1970). Retroviruses have evolved to select a tRNA from the intracellular milieu to function as a primer for reverse transcription (Mak et al., 1997; Marquet et al., 1995). A region on the viral genome, termed the primer binding site (PBS) is complementary to the 3’ terminal 18-nucleotides of the tRNA primer. The selection of a specific primer is conserved amongst different retroviruses (Mak et al., 1997; Marquet et al., 1995). Human immunodeficiency virus (HIV-1) preferentially selects tRNA\textsuperscript{Lys,3} as the primer for initiation of reverse transcription.

Previous studies from this laboratory and others have investigated the mechanism of primer selection through alteration of the PBS to be complementary to alternative tRNAs (Das, Klaver, and Berkhout, 1995; Li et al., 1994; Wakefield, Wolf, and Morrow, 1995). Mutation of the PBS to correspond to a variety of different tRNAs allows HIV-1 to transiently utilize these tRNAs for replication. However, all of the viruses revert to utilize the wild type tRNA\textsuperscript{Lys,3} following limited \textit{in vivo} replication. Further studies have revealed that a region upstream of the PBS designated as the A-loop is important for primer selection (Isel et al., 1995; Isel et al., 1993). The A-loop is postulated to interact with the anticodon of the tRNA primer during the initiation of reverse transcription. Alteration of both the A-loop and PBS to correspond to certain tRNAs results in the virus able to stably utilize these tRNAs for replication (Dupuy et al., 2003; Kang and Morrow,
A virus has been created that can utilize tRNA\textsuperscript{Lys\textsubscript{1,2}}, rather than tRNA\textsuperscript{Lys\textsubscript{3}} for replication. Although viruses can be forced to utilize tRNA\textsuperscript{Lys\textsubscript{1,2}} through mutations of the A-loop and PBS; however, this virus grows slower than the wild type virus (Kang, Zhang, and Morrow, 1999; Moore et al., 2004). The reason for the reduced replicative capacity of viruses forced to utilize tRNA\textsuperscript{Lys\textsubscript{1,2}} is not clear since previous studies have shown that both tRNA\textsuperscript{Lys\textsubscript{3}} and tRNA\textsuperscript{Lys\textsubscript{1,2}} are preferentially incorporated into HIV-1 virions. A recent study from this laboratory though has found that alteration of the codon use within the Gag protein prior to the Gag-pol frameshift site to favor the use of tRNA\textsuperscript{Lys\textsubscript{1,2}} can have significant impact on the replication of viruses that are forced to use tRNA\textsuperscript{Lys\textsubscript{1,2}} (Palmer et al., 2007). In this study, we found that alteration of the five lysine codons prior to the Gag-pol frameshift site resulted in an enhanced replication of virus that was forced to utilize tRNA\textsuperscript{Lys\textsubscript{1,2}} through alteration of the A-loop and PBS. The results of these studies then suggest that the primer selection process is linked to viral translation.

Since it is generally proven difficult to modulate the endogenous levels of tRNAs within eukaryotic cells due to toxicity, in previous studies, we have developed and utilized an \textit{in trans} complementation system in which to understand elements of the HIV primer selection process (Kelly, Palmer, and Morrow, 2003). In the first system, we utilized HIV-1 in which the PBS was altered to be complementary to yeast tRNA\textsuperscript{Phe}. Replication of this virus was achieved through the \textit{in trans} complementation of plasmids which encoded yeast tRNA\textsuperscript{Phe}. Using this system, we determined that nuclear to cytoplasmic transport of tRNA and inclusion into the translational cycle of the cell
facilitated the selection of tRNA as the primer for reverse transcription. In more recent studies, we have altered this system to allow for the selection of tRNA$^{\text{Lys,3}}$. For this system, we utilized *E. coli* tRNA$^{\text{Lys,3}}$ which differs enough within the 3’ terminal 18-nucleotides from mammalian tRNA$^{\text{Lys,3}}$ that a provirus in which the PBS was complementary to *E. coli* tRNA$^{\text{Lys,3}}$ was infectious only upon co-transfection of the plasmid encoding *E. coli* tRNA$^{\text{Lys,3}}$ (McCulley and Morrow, 2006). Recent studies have determined that alteration of the *E. coli* tRNA$^{\text{Lys,3}}$ to be identical to mammalian tRNA$^{\text{Lys,3}}$ accept for the 3’ terminal 18-nucleotides results in more efficient complementation. Thus, this *in trans* complementation system allows us to delineate important aspects of HIV-1 primer selection using tRNA closely related to mammalian tRNA$^{\text{Lys,3}}$.

In the current study, we have further investigated the link between viral translation and primer selection. For these studies, we have altered the lysine codon preference in HIV-1 Gag to be specific for tRNA$^{\text{Lys,1,2}}$. This necessitated altering the complete codon use of the Gag gene to the Gag-pol frameshift region. Alteration of all lysine codons prior to the Gag-pol frameshift region to be specific for tRNA$^{\text{Lys,1,2}}$, severely impacted on the production of infectious virus using the *in trans* complementation system. Furthermore, alteration of the five codons prior to the Gag-pol frameshift site to favor tRNA$^{\text{Lys,3}}$ resulted in enhanced replication. Results of these studies further strengthen the idea that viral translation and codon use with respect to tRNA$^{\text{Lys,1,2,3}}$ are important elements in preferential selection of tRNA$^{\text{Lys,3}}$ as the primer for HIV replication.
Materials and Methods

Tissue culture.

293T cells, JC53BL cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS) and 1% antibiotic (Gibco/BRL, Gaithersburg, MD).

Plasmid construction.

The proviral plasmid encoding the full-length HIV-1 genome (NL4) with a PBS altered to be complementary to the 3’-terminal 18 nucleotides of \( E. coli \) tRNA\(^{\text{Lys3}} \) was constructed as previously described (Yu, McCulley, and Morrow, 2007). The HIV-1 provirus with five codons for lysine previous to the Gag-pol frameshift region mutated to be specific for tRNA\(^{\text{Lys3}} \) (F3) or tRNA\(^{\text{Lys1,2}} \) (F5) were constructed as previously described (Palmer et al, Virology, in press). To modify the PBS of these provirus, a shuttle vector containing PBS complementary to \( E. coli \) tRNA\(^{\text{Lys3}} \) (Yu, McCulley, and Morrow, 2007) was digested with BssHII and HpaI enzymes in order to release an 868 bp fragment. This \( E. coli \) PBS containing fragment was then subcloned into pNL4-3(F5) or pNL4-3 (F3) to create proviral plasmids NL4-3EcPBS-F5 and pNL4-3-EcPBS-F3 respectively. An HIV-1 proviral genome was created in which all of the lysine codons from nucleotides 711 to 2006 (not encompassing the last five lysine codons previous to the Gag-pol frameshift site) were mutated to be specific for tRNA\(^{\text{Lys1,2}} \) codons. This gag gene fragment was synthesized (Blue Heron, Wisconsin) and subcloned into the \( \text{BssHII} \) and \( \text{ApaI} \) site of the wild type Ec PBS NL-4, ECPBS NL-4-F3 and Ec PBS NL-4-F5 to generate Ec PBS NL4 (BH), Ec PBS NL-4-F3 (BH) and EcPBS NL-4-F5 (BH).
The construction of *E. coli* tRNA^{Lys3} (MA) was previously described (McCulley and Morrow, 2006). All plasmids were screened with enzyme restriction and verified by DNA sequencing.

**DNA transfections.**

Complementation of HIV-1 proviral mutants were accomplished by co-transfection of tRNA-carrying plasmids with HIV-1 proviral mutants into 293T cells. Co-transfection was achieved by using a calcium-phosphate method. Briefly, 293T cells were seeded at a concentration of 2 x 10^5 cells per well in six-well plates. The cells were co-transfected with 500ng of proviral plasmid and increasing amounts of tRNA-carrying plasmid 24 h later. At approximately 7h post-transfection, the cells were washed once with 1× phosphate-buffered saline and supplied with fresh media. Supernatants were collected approximately 48 h post-transfection, centrifuged at 3,000 µg, and used in a JC53-BL assay to determine luciferase activity, which has been determined to correlate to the units of the infectious virus that is being tested.

**JC53-BL infection.**

Supernatants collected form co-transfections were used in a JC53-BL assay in order to determine infectious viral units (Moore et al., 2004). JC53-BL cells were seeded 24 h pre-infection. Infected cells were incubated for 2 h in 37°C incubator supplemented with 5% CO₂. After 2 h, DMEM with 10% FBS was added to each well and the cells were incubated for additional 48 h. To determine luciferase activity, cells were lysed using M-PER mammalian Protein Extraction Reagent (PIERCE, Rockford, IF) and approximately 20ul of each lysed sample were transferred to a microplate. Reporter Lysis Buffer (Promega, Madison, WI) was added to each sample in the microplate and the light
intensity was measured using a Tropix TR717 Microplate Luminometer (Applied Biosystems, Foster City, CA). Uninfected cells in wells represented background luciferase activity which was subtracted from all other samples. Relative Light Units (rLU) per ml were calculated by dividing the luciferase value by their corresponding dilutions.

**p24 Gag antigen ELISA.**

Virus production from co-transfection of proviral plasmids and *E. coli* tRNA<sup>Lys</sup><sup>3</sup> (MA) were monitored by p24 antigen levels using a commercial ELISA kit (Beckman Coulter, Miami, FL).
Results

In a recent study we found that alteration of the lysine codons near the Gag-pol frameshift site of HIV-1 had an impact on the capacity of the virus to utilize tRNA$^{\text{Lys1,2}}$ as the primer for reverse transcription (Palmer et al., 2007). Alteration of the five lysine codons to correspond that for tRNA$^{\text{Lys1,2}}$ resulted in a proviral genome (F5) that had enhanced replication similar to the wild type virus. In the recurrent study, we have made additional mutations in which the lysine codons prior to the Gag-pol frameshift site were altered to be specific for tRNA$^{\text{Lys1,3}}$ (F3) (Figure 1). An additional mutation was made in these viral genomes in which the PBS was altered to correspond to the 3’ terminal 18-nucleotides of \textit{E. coli} tRNA$^{\text{Lys3}}$. The resulting proviral genomes were named NL4-EcPBS, NL4-EcPBSF3 and NL4-EcPBSF5. For these studies, we have used an \textit{E. coli} tRNA$^{\text{Lys3}}$ that has been further altered such that the nucleotides within the anticodon stem loop now correspond to mammalian tRNA$^{\text{Lys3}}$, which resulted in an enhanced complementation of viruses the PBS complementary to \textit{E. coli} tRNA$^{\text{Lys3}}$ (Figure 2) (McCulley and Morrow, 2007).

We first analyzed the \textit{in trans} complementation of the different proviral genomes using \textit{E. coli} tRNA$^{\text{Lys3}}$ supplied \textit{in trans}. Following co-transfection, the released virus was analyzed for virus production (p24) and infectivity using the JC53-BL assay. There was no difference in the amount of virus produced from each proviral construct as determined by p24 antigen capture ELISA (Figure 2A). Consistent with our previous study, we found an increase in the production of infectious virus with increasing amounts of \textit{E. coli} tRNA$^{\text{Lys3}}$ plasmid provided \textit{in trans} (Figure 2B). The amounts of infectious virus from transfection of NL4-EcPBS peaked at approximately 500 nanograms of \textit{E. coli}
tRNA_{Lys,3} plasmid provided in trans. Modification of the 5 lysine codons prior to the Gag-pol frameshift had a significant effect on the production of infectious virus. Alteration of the codons to be specific for tRNA_{Lys,3} (NL4-EcPBSF3) resulted in increased levels of infectious virus production when co-transfected with the plasmid encoding *E. coli* tRNA_{Lys,3}. In contrast, proviral genomes in which the five codons were specific for tRNA_{Lys1,2} (NL4-EcPBSF5) produced less infectious virus than NL4-EcPBS or NL4-EcPBSF3.

Our next experiments were designed to determine the effects that a complete alteration of the lysine codons to be specific for tRNA_{Lys1,2} would have on production of infectious virus. For these studies, we had the remaining lysine codons in the HIV-1 Gag gene, up to the last 5 codons prior to the Gag-pol frameshift region, altered to be specific for tRNA_{Lys1,2} (Figure 3). We then combined this modification with the wild type sequence which contains three codons for tRNA_{Lys,3} and two for tRNA_{Lys1,2} prior to the Gag-pol frameshift (wild type; NL4-EcPBS-BH) and two additional genomes that contain either all five codons specific for tRNA_{Lys,3} (NL4-EcPBS-F3-BH) or all five specific for tRNA_{Lys1,2} (NL4-EcPBSF5-BH). We then compared the production of virus following complementation with the plasmid encoding *E. coli* tRNA_{Lys,3}. No significant difference was found with respect to virus production (as reassured by p24) for the different proviral constructs (data not shown). The production of infectious virus from proviral genomes with the alteration of codons to be specific for tRNA_{Lys1,2} resulted in a reduction in the production of infectious virus (Figure 4). Regardless of whether or not the last five lysine codons were altered to be specific for tRNA_{Lys,3} or tRNA_{Lys1,2}, the amounts of infectious virus plateaued at levels less than that for the corresponding proviral genomes without the
alteration in the lysine codons. Thus, alteration of the lysine codons to be specific for
\(\text{tRNA}^{\text{Lys}}_{1,2}\) had an impact on the capacity of the virus to be complemented with \(E.\ coli\)
tRNA\(^{\text{Lys}}_{3}\) provided\textit{ in trans}. 
Discussion

In the current study, we have utilized a novel in trans complementation system to further investigate of HIV-1 primer selection. For these studies, we utilized an HIV-1 proviral genome in which the PBS was mutated to be complementary to the 3’ terminal 18-nucleotides of E. coli tRNA^{Lys,3}. Infectivity of this proviral genome was dependent upon the co-expression of E. coli tRNA^{Lys,3} in transfected cells. In a recent study, we have found that the capacity of HIV-1 to utilize an alternative primer, tRNA^{Lys,1,2}, could be enhanced through modification of five lysine codons prior to the Gag-pol frameshift region (Palmer et al., 2007). In the current study, we have extended this work to show that alteration of the ratio of codons for tRNA^{Lys,3} and tRNA^{Lys,1,2} prior to the Gag-pol frameshift region could have significant effects on the generation of infectious virus. Mutating the five lysine codons prior to the Gag-pol frameshift the tRNA^{Lys,3} enhanced the complementation as evidenced by increased infectivity while alteration of the five codons to correspond to tRNA^{Lys,1,2} resulted in reduced complementation. We extended this work to analyze the effects of mutation of a lysine codon prior to the Gag-pol frameshift to correspond entirely to tRNA^{Lys,1,2}. Production of virus observed with this proviral genome, regardless of whether the five lysine codons prior to the Gag-pol frameshift were mutated, reduced production of infectious virus, indicating that the lysine codon ratios prior to the Gag-pol frameshift region can influence primer selection.

Previous studies have investigated the process of HIV-1 primer selection through modification of the PBS to be complementary to alternative tRNAs (Das, Klaver, and Berkhout, 1995; Li et al., 1994; Wakefield, Wolf, and Morrow, 1995). In some instances, modification of both the A-loop and PBS regions could correspond to the anticodon and
3’ terminal nucleotides of certain tRNAs has resulted in the capacity of the virus to utilize these tRNAs for replication (Dupuy et al., 2003; Kang and Morrow, 1999; Kang, Zhang, and Morrow, 1997; Kang, Zhang, and Morrow, 1999; Li et al., 2006; Ni and Morrow, 2006; Wakefield, Kang, and Morrow, 1996; Zhang et al., 1998). All of these viruses though, replicated with infectivity lower than the wild type virus (Wei et al., 2005). Indeed, a virus altered to facilitate the use of tRNA^{Lys}_1,2 could select and utilize this tRNA following \textit{in vitro} replication but replicated at a level much lower than that of the wild type virus (Palmer et al., 2007). In a recent study, we have found that alteration of the codons for lysine prior to the Gag-pol frameshift greatly enhanced the replication of this virus to levels approaching that of the wild type virus (Palmer et al., 2007). The results of these studies, as well as others, from this laboratory suggest a link between the viral translation and primer selection. To further explore this relationship, we have utilized a novel complementation system in which tRNAs provided \textit{in trans} were the intracellular selection by HIV-1. A previous studies from this laboratory have shown that \textit{E. coli} tRNA^{Lys}_3 can be modified to be nearly identical with mammalian tRNA^{Lys}_3 except for the 3’ terminal 18-nucleotides (McCulley and Morrow, 2006). Complementation using this \textit{E. coli} tRNA^{Lys}_3 is efficient and allows the production of infectious HIV-1. Previous studies have shown that expression of the modified \textit{E. coli} tRNA^{Lys}_3 in mammalian cells results in the aminoacylation of this tRNA and presumably inclusion into the host cell translational process (McCulley and Morrow, 2006). However, for the \textit{E. coli} tRNA^{Lys}_3 to be used during translation, it must also compete with the endogenous mammalian tRNA^{Lys}_3 for occupancy of ribosomes. At present, it is not clear how effectively \textit{E. coli} tRNA^{Lys}_3 is actually used for translation when expressed
in cells. To determine if we could influence primer selection, we constructed HIV genomes in which the codons for lysine were modified to be specific for tRNA$^{\text{Lys,3}}$ or tRNA$^{\text{Lys,1,2}}$ prior to the Gag-pol frameshift site. Interestingly, results demonstrate that favoring the use of codons specific for tRNA$^{\text{Lys,3}}$ prior to the Gag-pol frameshift site actually increased the production of infectious virus. The results of these studies then support the idea that during the process of ribosomal frameshifting to generate the Gag-pol protein there is a link with primer selection.

To further explore the relationship between viral translation and primer selection, we reconstructed the HIV-1 Gag gene prior to the Gag-pol frameshift so as to make all of the lysine codons specific for tRNA$^{\text{Lys,1,2}}$ (Figure 3). We then assessed the effect of this mutation in the HIV-1 genome on the production of infectious virus following complementation with *E. coli* tRNA$^{\text{Lys,3}}$. Regardless of whether or not the additional mutations of the five lysine codons prior to the Gag-pol frameshift site were specific for tRNA$^{\text{Lys,3}}$, we found less infectious virus generated using these proviral genomes. Thus, forcing the use of tRNA$^{\text{Lys,1,2}}$ during translation of HIV-1 Gag had an unexpected effect on the production of infectious virus. There are several explanations for this result. The first could be there was less virus produced from those genomes favoring the use of tRNA$^{\text{Lys,1,2}}$. The viral genomes with codons specific for tRNA$^{\text{Lys,1,2}}$ might exhaust the endogenous levels of tRNA$^{\text{Lys,1,2}}$ and thus inhibit viral translation. This seems unlikely since we found analysis of the p24 released following transfection, which is indicative of Gag and Gag-pol synthesis, was similar for virus with or without the bias for use of tRNA$^{\text{Lys,1,2}}$. Indeed, if tRNA$^{\text{Lys,1,2}}$ uses , tRNA$^{\text{Lys,3}}$ could be used with these codons with these codons from codon wobble. Second, it is possible that an intracellular pool of
tRNAs exist from primer selection by HIV-1. Previous studies from our laboratory have shown that certain tRNAs are favored over others as primers for selection by HIV-1 (Li et al., 2006; Ni and Morrow, 2006; Ni, Xu, and Morrow, 2007). Based on these results, we suggested that an intracellular pool of tRNAs exist with tRNA^{Lys,3} as the most prominent. The results of our studies suggest that translation of the viral genome (i.e., Gag) can impact this pool and effect primer selection. That is, the over use of tRNA^{Lys,1,2} by these mutated proviral genomes biased the tRNA^{Lys,3} to tRNA^{Lys,1,2} primer selection pool to favor that for tRNA^{Lys,1,2}. Increasing the numbers of tRNA^{Lys,1,2} codons then would result in more tRNA^{Lys,2} used in translation thus making the selection of tRNA^{Lys,3} more difficult. An alternative explanation could also be that with more use of tRNA^{Lys,1,2}, the more endogenous tRNA^{Lys,3} would be available for translation and thus compete with *E. coli* tRNA^{Lys,3} for use in translation, which would impact on the efficiency of primer selection. Since it has proven difficult to modulate the endogenous level of tRNAs in mammalian cells, future studies using this *in trans* complementation system with codon modified viral genomes will be needed to delineate the dynamics between viral translation and primer selection.
Acknowledgements

We thank members of the Morrow Laboratory for helpful comments. Adrienne Ellis is thanked for preparation of the manuscript. DNA sequencing was carried out by the UAB CFAR DNA Sequencing Core (AI 27767). CDM acknowledges MAR for helpful conversation. This work was supported by a grant from the NIH to CDM (AI34749).
References


Figure 1: HIV-1 proviral genomes with alteration in lysine codons prior to the Gag-pol frameshift junction.

Panel A. HIV proviral genome. The expression of Gag-pol occurs via a ribosomal frameshift. The region in which the lysine codons were modified is shown in grey.

Panel B. HIV-1 proviral genomes were modified such that the five lysine codons prior to the Gag-pol frameshift region were altered to be specific for tRNA\(^{Lys,3}\) (F3) or tRNA\(^{Lys,1,2}\) (F5). The resulting proviral genomes were also altered so the PBS would be complementary to the 3' terminal 18-nucleotides of \(E.\ coli\) tRNA\(^{Lys,3}\).

Panel C. \(E.\ coli\) tRNA\(^{Lys,3}\) modified to correspond to mammalian tRNA\(^{Lys,3}\). \(E.\ coli\) tRNA\(^{Lys,3}\) shares many common features with mammalian tRNA\(^{Lys,3}\). Recent studies have shown that modification of the nucleotides within the anticodon region did correspond to mammalian tRNA\(^{Lys,3}\) enhanced the production of infectious virus. Presented is a diagram of the \(E.\ coli\) tRNA\(^{Lys,3}\) which has been modified to correspond to mammalian tRNA\(^{Lys,3}\) though alterations in the anti-codon stem-loop (McCulley and Morrow, 2006).
Figure 2: Characterization of HIV-1 produced from transfection of wild type and codon modified proviral genomes.

Panel A. The wild type (NL4-EcPBS) and proviral genomes in which the five lysines prior to the Gag-pol frameshift were altered to be specific to tRNA_{Lys,3}^{EcoPBS} (NL4-EcPBSF3) or tRNA_{Lys,1,2}^{EcoPBS} (NL4-EcPBSF5) were transfected into 293T cells and the amount of virus produced by p24 antigen capture ELISA was determined.

Panel B. Determination of the amount of infectious HIV-1 produced from wild type and mutant proviral genomes co-transfected with the plasmid encoding *E. coli* tRNA_{Lys,3}^{EcoPBS}. 293T cells were co-transfected with 500ng proviral plasmids and with the EcoRNA_{Lys,3}^{EcoPBS} at 100ng, 250ng, 500ng, and 1000ng. Dilutions of collected supernatants that were acquired from co-transfections were used to infect the JC53-BL cell line which contains a luciferase gene under the transcriptional control of the HIV-1 LTR. Background luciferase activity obtained from mock-transfected culture was subtracted from each sample. The data denote means +/- standard deviations derived from three independent experiments.
Figure 3: Construction of HIV-1 genomes in which lysine codons prior to the Gag-pol junction have been modified to use tRNA\textsubscript{Lys1,2}. The lysine codons prior to the Gag-pol frameshift were modified to be specific for tRNA\textsubscript{Lys1,2} (denoted by arrows). In order to do this, the Gag gene was synthesized with the corresponding lysine codons all altered to be specific for tRNA\textsubscript{Lys1,2} (AAG). The codon modified Gag genes (designated as BH) were then cloned into the wild type genome (NL4-EcPBSBH) resulting in a proviral clone that contained lysine modified codons within Gag prior to the five preceding the Gag-pol frameshift junction. Two additional proviral constructs were made in which the five lysine codons preceding the Gag-pol frameshift site were altered to correspond to tRNA\textsubscript{Lys1,2} (NL4-EcPBS F3) or proviral clones in which the five lysine codons preceding the Gag-pol frameshift site were altered to correspond to tRNA\textsubscript{Lys1,2} (NL4-EcPBS F5).
Figure 4: Complementation of the proviral plasmids with plasmid that encodes *E. coli* tRNA<sup>Lys<sub>3</sub></sup> (MA).

**Panel A.** Complementation of the NL4-EcPBS and NL4-EcPBS BH with plasmids that encodes *E. coli* tRNA<sup>Lys<sub>3</sub></sup>. 293T cells were co-transfected with 500ng proviral plasmids and with the RNA plasmids that were titrated in at the quantities of 100ng, 250ng, 500ng, and 1000ng. Dilutions of collected supernatants that were acquired from co-transfection were used to infect the JC53-BL cell line, followed by determination of luciferase activity.

**Panel B.** Complementation of NL4-EcPBSF3 and NL4-EcPBSF3BH by *E. coli* tRNA<sup>Lys<sub>3</sub></sup>.

**Panel C.** Complementation of NL4-EcPBS F5 and NL4-EcPBS F5 BH by *E. coli* tRNA<sup>Lys<sub>3</sub></sup> provided *in trans*. 
DISCUSSION AND CONCLUSIONS

The replication cycle of HIV-1 is characterized by the process of reverse transcription to convert the RNA genome into a double strand DNA copy, a mechanism that is shared by all retroviruses. As a potential therapeutic target, extensive research has been centered on reverse transcription. Reverse transcription is initiated by the binding of a cellular tRNA$^{\text{Lys,3}}$ to the PBS of the viral genomic RNA. To further understand the process of primer selection during reverse transcription, it would be advantageous to manipulate the tRNA primer. However, it has proven to be quite difficult to manipulate endogenous tRNA levels in mammalian cells due to a tight regulation in the biogenesis of tRNAs as a means to control translation. To circumvent this issue, the current research has used a single round complementation system that allows tRNA to be supplied in trans and to be selected as a primer. This system relies on the use of *E.coli* tRNA$^{\text{Lys,3}}$, which maintains many of the features of the mammalian tRNA$^{\text{Lys,3}}$ but has different 3’ end 18 nucleotides.

*E.coli* tRNA$^{\text{Lys,3}}$ is an ideal candidate to examine the role of specific regions or nucleotides of tRNA in primer selection and reverse transcription because a combination of two posttranscriptional anticodon modifications distinguishes the *E.coli* and mammalian tRNA$^{\text{Lys,3}}$ from other tRNA$^{\text{Lys}}$ species: wobble position 34 is modified by 5-methoxycarbonylmethyl-2-thiouridine (mcm$^5$U34) in human tRNA$^{\text{Lys}}$ and a closely related 5-methylaminomethyl-2-thiouridine (mmn$^5$s$^2$U34) in *E.coli* tRNA$^{\text{Lys,3}}$. Adenosine-
adjacent to the anticodon, is modified by 2-methylthio-N6-threonylcarbamoyladenosine \( (m^2t^6A37) \) in tRNA\(^{Lys,3}\) and by \( t^6A37 \) in the \( E.coli \) tRNA\(^{Lys,3}\). The \( E.coli \) tRNA\(^{Lys,3}\) anticodon stem and the complete anticodon loop are identical to that of human tRNA\(^{Lys,3}\) \( (173) \). On the other hand, there is enough difference between the mammalian tRNA\(^{Lys,3}\) and \( E.coli \) tRNA\(^{Lys,3}\) 3’ terminal 18 nucleotides to allow for specific base-pairing between the PBS and tRNA, excluding the endogenous tRNA\(^{Lys,3}\) priming. Wild type HIV-1 utilizes tRNAs containing \( m^1A58 \) as primers for reverse transcription. In addition, the HIV-1 genome contains PBS of 18 nucleotides in length, which suggests that residue \( m^1A58 \) serves as a termination signal for +sssDNA synthesis. Thus, it is possible that mutation of \( m^1A58 \) in the tRNA primer may lead the +sssDNA to be synthesized beyond \( m^1A58 \) site and thus, inhibit the replication of the virus. Design of the A58U mutation is based on the fact that tRNA 1-adenosine methyl-transferases are specific for an A in position 58 and not other bases \( (200) \). Renda et al \( (164) \) suggested that the A58U might not influence the capacity of this mutated tRNA to be aminoacylated because this one point mutation cannot change the overall structure of the tRNA. The fact that Renda et al also reported “breakthrough” HIV-1 replication in some tRNA\(^{Lys,3}\)A58U expressing cell cultures leads us to doubt the importance of tRNA \( m^1A58 \) in HIV-1 replication. That is, if \( m^1A58 \) plays a very important role in terminating +sssDNA synthesis and leading to the second jump, it is not clear how the virus completes reverse transcription in tRNA\(^{Lys,3}\)A58U expressing cell cultures. There is some controversy concerning the precise +sss DNA termination signal. Auxilien and his collaborators \( (201) \) show three RT termination sites due to modified nucleotides \( m^1A58, Tm54, \) and \( m^2t^6A37 \) in the reverse transcription of natural tRNA\(^{Lys,3}\), while these stops
are not observed with synthetic tRNA\textsuperscript{Lys,3}. Ben-Artzi et al (112) reported that two determinants serve as stop signals for +sss DNA synthesis. One is the methylated A58 residue in tRNA\textsuperscript{Lys,3}, the other one is the secondary structure of the PBS sequence.

Based on previous research demonstrating that the 1-adenosine methyltransferase is nucleotide specific, mutation of A58 to U will abolish the methylation of the nucleotide. If the methylated A in position 58 of tRNA\textsuperscript{Lys,3} serves as the stop signal for synthesis of +sssDNA, when we mutate the A58 to U, there will be no stop signal and the reverse transcription will continue to reverse transcribe the following sequence of the tRNA primer. This will disrupt the second DNA transfer and the reverse transcription can not be completed. In the current research, we examined the ability of \textit{E.coli} tRNA\textsuperscript{Lys,3} A58U to complement the replication of \textit{E.coli} PBS HIV-1. We compared the complementation capacity of \textit{E.coli} tRNA\textsuperscript{Lys,3} A58 U to wild type \textit{E.coli} tRNA\textsuperscript{Lys,3} on the replication of \textit{E.coli} PBS HIV-1. Our result suggested that mutations in A58 to U did not affect the capacity of this tRNA primer to complement viral replication. This result demonstrated that the post-transcriptional modification of A58 is not or only partially served as the stop signal for plus strand strong stop DNA synthesis. This result is consistent with the possibility that additional features of the tRNA\textsuperscript{Lys,3} primer are more important than the modified bases for the termination of plus-strand synthesis (112). Isel et al proposed a model for the initiation complex formed by genomic RNA and tRNA\textsuperscript{Lys,3} primer. As a consequence of this tRNA: RNA genome interaction, the tRNA\textsuperscript{Lys,3} structure is disrupted and new intramolecular bonds are formed. One of the important new RNA: RNA interactions are between part of the TΨC loop and the 5’ terminal within the tRNA primer. We apply this model to the interaction between the primer tRNA and the plus strand strong stop
DNA. Our assumption is that when the 3’terminal 18 nucleotides are complemented with the 3’terminal of plus strand strong stop DNA, a newly secondary structure will form between the 5’terminal nucleotides and part of the TΨC stem-loop within the tRNA primer. This new formed secondary structure may serve as the stop signal for the plus strand strong stop DNA synthesis. In order to address our assumption, we made several mutations within the *E. coli* tRNA<sup>Lys,3</sup> to strengthen or weaken the secondary structure. We provided these mutated *E. coli* tRNA<sup>Lys,3</sup> to check their ability to complement the replication of HIV-1 which is forced to specifically use *E. coli* tRNA<sup>Lys,3</sup> as a primer to initiate reverse transcription. One of the important new secondary structures is between nucleotides 53 and 54 and the first two nucleotides of tRNA<sup>Lys,3</sup> (151). The first mutation targeted the nucleotide at position 54 which is a T. Two mutations were made in which this thymidine was changed to a G or C respectively. We also made an additional mutation in which the TG at positions 54 and 53 were altered to Cs. The mutations we made are designated as *E. coli* tRNA<sup>Lys,3</sup> T54G, *E. coli* tRNA<sup>Lys,3</sup> T54C, and *E. coli* tRNA<sup>Lys,3</sup> TG5453/CC respectively. Surprisingly, our data revealed that *E. coli* tRNA<sup>Lys,3</sup> T54G complemented Ec PBS HIV-1 replication to a similar level as wild type *E. coli* tRNA<sup>Lys,3</sup>. While, *E. coli* tRNA<sup>Lys,3</sup> T54C and *E. coli* tRNA<sup>Lys,3</sup> TG5453/CC could not complement Ec PBS HIV-1 replication. *E. coli* tRNA<sup>Lys,3</sup> T54G is predicted to weaken the newly formed secondary structure, and *E. coli* tRNA<sup>Lys,3</sup> T54C and *E. coli* tRNA<sup>Lys,3</sup> TG5453/CC should strengthen it.

We also examined the aminoacylation level of these mutated *E. coli* tRNA<sup>Lys,3</sup> in mammalian cells, since capacity to be aminocylated indicates that the tRNA is functional, and only functional tRNA can be selected and used as a primer by the virus. Northern
blot analysis revealed both T54G and TG5453CC E. coli tRNA\[^{Lys,3}\] mutants are predominantly aminoacylated within mammalian cells; in contrast, the T54C mutation in E. coli tRNA\[^{Lys,3}\] was poorly aminoacylated and consequently expressed at a lower level than the other mutants. The inability of be aminoacylated in mammalian cells explains the incapability of E. coli tRNA\[^{Lys,3}\] T54C to complement the replication of E. coli PBS HIV-1 replication. Beerens et al established that the sequence of TΨC stem loop in the tRNA primer interacts with the HIV-1 genome within the U5 region (PAS). It is highly possible that the mutation of T54G in E. coli tRNA\[^{Lys,3}\] disrupts the interaction between the TΨC stem-loop and PAS region in the HIV-1 genome. However, we cannot rule out the possibility that the importance of single nucleotide in TΨC loop might be due to its interaction with viral proteins. In vitro studies (150) indicate that there is direct interaction between RT and the tRNA TΨC loop.

In summary, from the results of our studies, we conclude that the post-transcriptional modification at nucleotide 54 is not essential for function of tRNA\[^{Lys,3}\] in HIV-1 replication. Nucleotides within the TΨC stem loop are also important for tRNA: HIV-1 genome RNA interactions in initiation of reverse transcription. We found that one mutant, TG5453CC, could undergo aminoacylation, indicating that the tRNA structure was intact; however, it did not complement HIV-1 replication even though this mutant would be predicted to have a favorable interaction in the initiation complex between tRNA and HIV-1 RNA. A second mutant, T54G, which would be predicted to not favor the tRNA: HIV-1 genome RNA interaction in the initiation complex was fully functional for complementation. From these results, we conclude that a complex interaction between the HIV-1 viral genome in tRNA\[^{Lys,3}\] probably involves multiple, complex RNA:
RNA interactions during primer selection and reverse transcription. The absolute base pair complementary are not necessary for these interactions; consistent with the idea the RNA structures are probably dynamic during HIV-1 reverse transcription.

The Relationship between Viral Translation and Primer Selection

Previous research already demonstrated that \( E. coli tRNA^{\text{Lys, 3}} \) was able to complement the replication of HIV-1 which was constructed to contain a PBS sequence specifically complementary to 3’prime 18 nucleotides of E.coli tRNA\(^{\text{Lys, 3}}\). However, the resulting virus has much lower infectivity compare to that of wild type virus. McCulley et al recently found that the complementation of \( E. coli \) tRNA was enhanced if the nucleotides within the variable region and the anticodon stem of \( E. coli \) tRNA were made to correspond to mammalian tRNA\(^{\text{Lys, 3}}\). The mutated \( E. coli tRNA^{\text{Lys, 3}} \) was designated as \( E. coli \) tRNA (MA). Thus, in the current study, we utilized \( E. coli tRNA^{\text{Lys, 3}} \) (MA), instead of \( E. coli tRNA^{\text{Lys, 3}} \), in the complementation system to achieve enhanced complementation.

To understand the preferences for selection of specific tRNAs for HIV-1 replication, we have utilized a genetic approach to generate viruses in which the PBS has been altered to be complementary to other tRNAs. Previous studies from our laboratory have utilized this approach to generate HIV-1 which stably utilized tRNA\(^{\text{His}}\), tRNA\(^{\text{Glu}}\), tRNA\(^{\text{Met}}\), and tRNA\(^{\text{Lys1, 2}}\). Interestingly, the codons for lysine, methionine, histidine and glutamic acid were present near the Gag-pol frameshift region of HIV-1. Previous studies have shown that when the translational frameshifting occurs during synthesis of HIV-1 Gag-pol, ribosomes stall near the Gag-pol junction. During the pause in translation the ribosomes could become stalled over codons on the viral mRNA. Aminoacylated tRNA
entering the stalled ribosome, would be rejected, and following disassociation from the ribosome, would become a substrate for peptidyl-tRNA hydrolase to recycle the tRNA for inclusion in the protein synthesis cycle. We postulate, then, that the ribosomal pausing that occurs during frameshifting results in a local increase of tRNAs that could be captured as primers for retrovirus replication. For HIV-1, it is possible at this time that the lysyl-synthetase is occupied with mainly tRNA^{Lys,3} which would also facilitate the capture of this tRNA. As in the case of our mutants in Gag-pol, if we alter the codon usage to favor tRNA^{Lys,1,2}, the synthetase would have an overabundance of tRNA^{Lys,1,2} to facilitate capture. However, the mechanism by which the virus might acquire the tRNA from the synthetase is unclear and will require further study.

To further pursue the concept of a connection between viral translation and lysine codon use within Gag-pol and tRNA primer selection, the lysine amino acid sequence upstream of the Gag-pol frameshift region was examined. Among the last five codons for lysine upstream of the frameshift site, three of the five lysine codons were specific for the tRNA^{Lys,3} isoacceptor whereas two were specific for tRNA^{Lys,1,2}. In the current study, we have altered the last five codons prior to the Gag-pol junction to be specific for tRNA^{Lys,1,2} (F5) or to be specific for tRNA^{Lys,3} (F3) to determine if the five codons for lysine prior to the Gag-pol protein were influencing the process of primer selection. An additional mutation was made in these viral genomes in which the PBS was altered to correspond to the 3’terminal 18-nucleotides of E.coli tRNA^{Lys,3}; this tRNA has been further altered such that the nucleotides within the anticodon stem loop now correspond to mammalian tRNA^{Lys,3}. We analyzed the complementation of these different proviral genomes using E.coli tRNA^{Lys,3} supplied in trans. Following co-transfection, the released
virus was analyzed for infectivity using the JC53-BL assay. Consistent with our previous study, we found that modification of the 5 lysine codons prior to the Gag-pol frameshift had a significant effect on the production of infectious virus. Alteration of the codons to be specific for tRNA$^{\text{Lys},3}$ (NL4-EcPBS-F3) resulted in increased levels of infectious virus production when co-transfected with the plasmid encoding *E.coli* tRNA$^{\text{Lys},3}$ (MA). In contrast, proviral genomes in which the five codons were specific for tRNA$^{\text{Lys},1,2}$ (NL4-EcPBS-F5) had lower complementation than the NL4-EcPBS or NL4-EcPBS-F3. The results of these studies support the idea that during the process of ribosomal frameshifting to generate the Gag-pol protein there is a link between primer selection and viral protein translation.

To further explore the relationship between viral translation and primer selection, we reconstructed the HIV-1 Gag gene prior to the Gag-pol frameshift so as to make all of the lysine codons specific for tRNA$^{\text{Lys},1,2}$. This experiment was designed to determine the effects that a complete alteration of the lysine codons to be specific for tRNA$^{\text{Lys},1,2}$ would have on production of infectious virus. We also combined this modification with the wild type sequence which contains three codons for tRNA$^{\text{Lys},3}$ and two for tRNA$^{\text{Lys},1,2}$ prior to the Gag-pol frameshift (wild type) and two additional genomes that contain either all five codons specific for tRNA$^{\text{Lys},3}$ or all five specific for RNA$^{\text{Lys},1,2}$. We then assessed the effect of this mutation in the HIV-1 genome on the production of infectious virus following complementation with *E.coli* tRNA$^{\text{Lys},3}$. Results showed that alteration of all lysine codons prior to the Gag-pol frameshift region to be specific for tRNA$^{\text{Lys},1,2}$ severely impacted on the production of infectious virus using the in trans complementation system.

The production of infectious virus form proviral genomes with the alterations of codons
to be specific for tRNA\textsubscript{Lys\textsubscript{1,2}} resulted in a reduction in the production of infectious virus. Regardless of whether or not the last five lysine codons were altered to be specific for tRNA\textsubscript{Lys\textsubscript{3}} or tRNA\textsubscript{Lys\textsubscript{1,2}}, the amounts of infectious virus plateaued at level less than that for the corresponding proviral genomes without the alteration in the lysine codons. Thus, forcing the use of tRNA\textsubscript{Lys\textsubscript{1,2}} during translation of HIV-1 Gag had an unexpected effect on the production of infectious virus. There are several explanations for this result. The first could be there was less virus produced from those genomes favoring the use of tRNA\textsubscript{Lys\textsubscript{1,2}}. The viral genomes with codons specific for tRNA\textsubscript{Lys\textsubscript{1,2}} might exhaust the endogenous levels of tRNA\textsubscript{Lys\textsubscript{1,2}} and thus inhibit viral translation. This explanation seems unlikely since the analysis of the p24 released following transfection, which is indicative of Gag and Gag-pol synthesis, did not show any significant difference. Furthermore, tRNA\textsubscript{Lys\textsubscript{3}} could be used as a wobble. Second, it is possible that the over use of tRNA\textsubscript{Lys\textsubscript{1,2}} by these mutated proviral genomes biased the tRNA\textsubscript{Lys\textsubscript{3}} to tRNA\textsubscript{Lys\textsubscript{1,2}} in primer selection pool to favor that for tRNA\textsubscript{Lys\textsubscript{1,2}}. This possibility would further support the idea that the use of tRNA in translation is important for primer selection. Increasing the numbers of tRNA\textsubscript{Lys\textsubscript{1,2}} codons within Gag gene would result in more tRNA\textsubscript{Lys\textsubscript{1,2}} used in translation thus making the selection of tRNALys\textsubscript{3} more difficult. An alternative explanation could be that with more use of tRNA\textsubscript{Lys\textsubscript{1,2}}, the more endogenous tRNA\textsubscript{Lys\textsubscript{3}} would be available for translation and thus compete with \textit{E.coli} tRNA\textsubscript{Lys\textsubscript{3}} for use in translation, which might also impact on the efficiency of primer selection.

In order to further explore the link between viral protein translation and primer selection, studies could examine additional HIV-1 mutants designed to use alternative tRNAs. For example, previous studies have shown that HIV-1 can use tRNA\textsubscript{Met} as the
primer for reverse transcription, but the virus grows slowly compared to wild type. Only one methionine is found prior to the Gag-pol junction. Based on a link between translation of Gag-pol and primer selection, it might be possible to enhance the replication of the virus designed to select tRNA$^{\text{Met}}$ by altering codons near the Gag-pol frameshift to correspond to methionine. If the virus infectivity are enhanced, this result would support the model for primer selection.

Finally the initiation of HIV-1 reverse transcription is an excellent target for drug design, molecules mimicking tRNA$^{\text{Lys,3}}$ would be predicted to inhibit HIV replication by either redirecting the initiation site of reverse transcription or by out competing the tRNA primer for the viral PBS or RT. Elucidation of elements within the tRNA primer critical for primer selection and use in HIV-1 will help to determine if we can design such new inhibitors of HIV-1 replication without disrupting host cell translation.
# LIST OF REFERENCES


10. Mervis, R.J., Ahmad, N., Lilleyhoj, E.P., Raum, M.G., Salazar, F.H.R., Chan, H.W. and Venkatesan, S.  The gag gene products of human immunodeficiency virus type 1: alignment within the gag open reading frame, identification of posttransla-


80. Ulrich, S., Anton, L.C., Cox, J.H., Bour, S., Bennink, J. R., Orlowski, M., Strebel, K., and Yewdell, J.W. CD4 glycoprotein degradation induced by Human Immu-


102. Lapham, C.K., Ouyang, J., Chandrasekhar, B., Nguyen, N.Y., Dimitrov, D.S.,
Golding, H.: Evidence for cell-surface association between fusin and the CD4-

103. Trkola, A., Dragic, T., Arthos, J., Binley, J.M., Olson, W.C., Allaway, G.P., et al.: CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-

induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine

coreceptors is required for infections by human immunodeficiency virus type 1. *J.


107. Melikyan, G.B., Markosyan, R.M., Hemmati, H., Delmedico, M.K., Lambert,
D.M., and Cohen, F.S. Evidence that the transition of HIV-1 gp41 into a six-helix
2000. 151:413-23

man, M., and Hope, T.J. Visualization of the intracellular behavior of HIV in liv-

109. Panganiban, A.T., and Fiore, D. Ordered interstrand and intrastrand DNA trans-

*Science.* 1990. 250:1227-33

111. Gotte, M., Li, X.G., and Wainberg, M.A. HIV-1 reverse transcription: A brief
overview focused on structure-function relationships among molecules involved

A. 1996. Molecular analysis of the second template switch during reverse tran-
scription of the HIV RNA template. *Biochemistry.* 35:10549-10557


114. de Noronha, C.M., Sherman, M.P., Lin, H.W., Cavrois, M.V., Moir, R.D., Gold-
man, R.D., Greene, W.C. Dynamic disruptions in nuclear envelope architecture
and integrity induced by HIV-1 Vpr. *Science.* 294:1105-8


127. Le Rouzic, E. et al. Docking of HIV-1 Vpr to the nuclear envelope is mediated by the interaction with the nucleoporin hCG1. J.Biol.Chem. 1998. 9:887-91


136. Cooney, A.J., Tsai, S.Y., O'Malley, B.W., Tsai, M.J. Chicken ovalbumin upstream promoter transcription factor binds to a negative regulatory region in the human immunodeficiency virus type 1 long terminal repeat. J. Virol. 1991. 65:2853-60


114


156. Kang, S.M., Wakefield, J.K., Morrow, C.D. Mutations in both the U5 region and the primer-binding site influence the selection of the tRNA used for the initiation of HIV-1 reverse transcription. *Virology.* 1996. 222:401-14


159. Kang, S.M., Zhang, Z., Morrow, C.D. Identification of a sequence within U5 required for human immunodeficiency virus type 1 to stably maintain a primer binding site complementary to tRNA(Met). *J. Virol.* 1997. 71:207-17


reverse transcription and inhibits replication of Human Immunodeficiency virus type I. J. Virol. 2001. 75:9671-8


Berkhout, B. Structure and function of the human immunodeficiency virus leader RNA. Prog. Nucleic Acid Res. Mol. Biol. 1996. 54:1-34

Beerens, N., Klaver, B., and Berkhout, B. A structure RNA motif is involved in correct placement of the tRNALys,3 primer onto the human immunodeficiency virus genome. J.Virol. 2000. 74:2227-38


