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Coupling Selection Of The HIV- 1 tRNA Primer Used For Reverse Transcription With Viral Translation And Encapsidation

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ABSTRACT

HIV-1 belongs to the *Retroviridae* family. A hallmark of retroviral replication is the process of reverse transcription. Viral reverse transcriptase requires placement of a cellular tRNA primer onto the primer binding site (PBS) in order to initiate conversion of the viral RNA genome into a double stranded DNA copy. Mutation of the HIV-1 PBS forces the virus to utilize the non-cognate primer. Even though extensive studies have been done over the past 15 years, the mechanism of HIV-1 primer selection has yet to be completely elucidated.

HIV-1 captures free tRNA during a yet undefined step in viral replication. However, our hypothesis that primer selection occurs at the level of translation is supported by data involving the HXB2^{yPhe} virus. Moreover, encapsidation of viral genomic RNA has also been linked with translation. In order to further investigate this relationship, we constructed proviruses in which the PBS was altered to be complementary to either elongator methionine (tRNA$^{Met(e)}$) (HXB2-Met(e))or initiator methionine (tRNA$^{Met(i)}$) (HXB2-Met(i)). The tRNAs interact with a completely different set of host cell proteins and are involved in separate phases of translation. Transfection of HXB2-Met(i) produced fewer and less infectious virus. It was unable to productively infect SupT1 cells. However, it was noticed that this virus had an inadvertently

introduced AUG codon in its PBS sequence. Therefore, a point mutation was introduced to alter the AUG to GUG (HXB2-Met(i) AG). This virus was able to productively infect SupT1 cells. Moreover, analysis of integrated proviral sequence revealed that it continuously selected $tRNA^{Met(i)}$ for use in reverse transcription.

A model is presented which suggests that this acquisition of tRNA occurs as a result of ribosomal pausing at the *gag-pol* frameshift site. This model is supported by data that shows that codon usage in this region affects the stability of HIV-1 PBS mutants. Therefore, in addition to the PBS and A-loop, we have identified a third region of viral RNA that plays a role in primer selection. In order to further investigate the relative contribution of the PBS, A-loop and the frameshift region in HIV-1 primer selection we constructed a series of mutants. Three groups of viruses were separated based on PBS complementarity to $tRNA^{Lys1,2}$, $tRNA^{Lys3}$, or $tRNA^{Met}$. Different combinations of A-loop and frameshift region mutations were introduced into the various proviral clones within the particular groups. The data obtained suggest that the frameshift region influences replication, although the specificity of primer is determined by the PBS and A-loop. Overall, the research presented in this dissertation has built upon previous findings and has linked primer selection, viral genomic RNA translation and encapidation.

DEDICATION

За Тајка, Мајчицу, Мацу и мога деда Жику

"*Veni Vidi Vici*"

-Iulius Caesar

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Fortitudo et decus!

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LIST OF ABBREVIATIONS

A

A site	aa-tRNA binding site
AIDS	acquired immunodeficiency syndrome
A-loop	polyadenosine loop in HIV
AMP	adenosine monophosphate
AP-1	activator protein-1 (transcriptional factor)
APC	antigen presenting cell
APOBEC3G	apolipoprotein B mRNA-editing enzyme catalytic polypeptidelike 3G
ARM	arginine-rich motif
ATG	translation start codon in DNA
ATP	adenosine triphosphate

B

C

CA	capsid domain of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}pol}$
CAp24	capsid protein
CCD	central core domain
CCHC	Cys-X2-Cys-X4-His-X4-Cys zinc-finger motif
CCR5	CC chemokine binding receptor

Cdk9	cellular cyclin-dependent kinase 9
cDNA	DNA molecule made as a copy of mRNA
cPTT	central polypurine tract
CTD	C-terminal domain
Crm1	chromosome maintenance region 1 (see Exportin 1)
CTL	cytotoxic T lymphocyte
CTP	cytosine triphosphate
CTS	central termination sequence
CypA	cyclophylin A
D	
DDDP	DNA dependent DNA polymerase
DDRP	DNA dependent RNA polymerase
DIS	dimer-initiation site
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double stranded DNA
E	
E site	ribosomal exit site
*Ec*tRNA^{Lys3}	*Escherichia coli* tRNA^{Lys3}
eEFs	eukaryotic elongation factors
eIFs	eukaryotic initiation factors
ELISA	Enzyme-Linked ImmunoSorbent Assay
env	envelope gene

Env	see gp160
ER	endoplasmic reticulum
eRF1	eukaryotic release factor
Ets	E26 transforming specific (transcription factor)
F	
FDA	Federal Drug Administration
FEN-1	flap endonuclease 1
G	
gag	group-specific antigen gene
Gag	*gag* encoded protein precursor
Gag-pol	*gag* and *pol* encoded protein precursor
GAP	GTPase-activating protein
GDP	guanosine diphosphate
gp160	*env*-encoded glycoprotein precursor
gp120	surface domain (SU)
gp41	transmembrane domain (TM)
GTF	general transcription factors
GTP	guanosine triphosphate
H	
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HTLV	human T cell leukemia virus

HTLV-1	human T cell leukemia virus type 1
HTLV-2	human T cell leukemia virus type 2
HTLV-3	human T cell leukemia virus type 3 (see HIV-1)
I	
Ig	immunoglobulin
IL-2	interleukin-2
IN	integrase enzyme
INp32	see IN
IRES	internal ribosome entry site
J	
JC53-BL	infectivity assay
K	
kDa	kilodalton
L	
LAV	lymphadenopathy associated virus (see HIV-1)
LAV-2	lymphadenopathy associated virus type 2 (see HIV-2)
LBP-1	leader binding protein 1
LTR	long terminal repeat
LysRS	lysyl-tRNA synthetase
M	
MA	matrix domain of domain of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}pol}$
MAp17	matrix protein
MHC-I	major histocompatibility complex class I

MHC-II	major histocompatibility complex class II
MHR	major homology region
mRNA	messenger RNA
msvRNA	multuply spliced viral RNA
MuLV	murine leukemia virus
MVB	multivesicular body
N	
N/A	not applicable
NC	nucleocapsid domain of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}pol}$
NCp7	mature nucleocapsid protein
NCp15	nucleocapsid protein precursor
NTD	N-terminal domain
Nef	negative regulatory factor
NES	nuclear export signal
NFAT-1	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B (transcription factor)
NHEJ	non-homologous end-joining
NLS	nuclear localization signal
NPC	nuclear pore complex
NRE	negative regulatory element
nt	nucleotide
O	
ORF	open reading frame

P

P site	ribosomal peptydyl-tRNA binding site
pA	poly A stem loop
PAB	poly(A) binding protein
PAS	primer activation signal
PBMC	peripheral blood mononuclear cell
PBS	primer binding site
PIC	preintegration complex
pol	polymerase gene encoding catalytic proteins
Pol	*pol*-encoded protein precursor
poly(A)	polyadenylation
PPT	polypurine tract
PR	protease
PRp11	protease monomer
$Pr55^{Gag}$	see Gag
$Pr160^{Gag\text{-}pol}$	see Gag-pol
PTAP	Pro-Thr-Ala-Pro motif of p6
P-TEFb	positive-transcriptional elongation factor

Q

R

RanGTP	Ran guanosine triphosphatase
RDDP	RNA dependent DNA polymerase
Ref1	human restriction factor

rER	rough ER
Rev	regulator protein
rev	
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RRE	Rev recognition sequence
RT	reverse transcriptase heterodimer
RTp51	p51 monomer of RT
RTp66	p66 monomer of RT
RTC	reverse transcription complex
S	
SIV	simian immunodeficiency virus
SIVcpz	SIV strain from chimpanzee
SIVcpz*Ptt*	SIVcpz infecting the *Pan Troglodytes Troglodytes* chimpanzee subspecies
SIVsm	SIV infecting sooty mangabeys (*Cercocebus atys*)
Sp1	transcription factor specificity 1
-sssDNA	minus strand strong stop DNA
+sssDNA	plus strand strong stop DNA
ssvRNA	singly spliced viral RNA
SU	surface glycoprotein, part of Env heterodimer
SupT1 cells	human caucasian T cell lymphoma cells
T	
TAR	trans-acting response element

Tat	transactivator of transcription protein
tat	tat gene
TATA box	TATA sequence
TBP	TATA-binding protein
TFIID	transcription factor II D
TM	transmembrane protein, part of Env heterodimer
tRNA	transfer RNA
$tRNA^{Asn}$	asparagine tRNA
$tRNA^{His}$	histidine tRNA
$tRNA^{Ile}$	isoleucine tRNA
$tRNA^{Lys}$	lysine tRNA
$tRNA^{Met}$	see $tRNA^{Met(e)}$
$tRNA^{Met(e)}$	elongator methionine tRNA
$tRNA^{Met(i)}$	initiator methionine tRNA
$tRNA^{Phe}$	phenylalanine tRNA
293T	human embryonic kidney cells
U	
U3	unique 3' region, part of LTR
U5	unique 5' region, part of LTR
usvRNA	unspliced viral RNA
USF	upstream stimulatory factor
UTR	untranslated region
V	

Vif	viral infectivity factor protein
vif	vif gene
VLP	virus-like particle
Vpr	viral protein R
vpr	vpr gene
Vpu	viral protein U
vpu	vpu gene
vRNA	HIV-1 viral RNA
vgRNA	viral genomic RNA

W

X

Y

$ytRNA^{Phe}$	yeast phenylalanine tRNA

Z

INTRODUCTION

Identification of HIV as the Cause of AIDS

Without even knowing it, physicians in New York and Los Angeles came face to face with clinical manifestations of acquired immunodeficiency syndrome (AIDS) in 1981 [1-4]. Previously healthy homosexual men started suffering from Kaposi's sarcoma, a rare cancer which typically affects elderly men of Mediterranean or Jewish heritage, organ transplant recipients, or young adult African men [3, 5]. Additionally, AIDS was characterized by generalized lymphadenopathy and opportunistic infections such as mucosal candidiasis and *Pneumocystis carinii* pneumonia [1, 3, 4]. Moreover, patients with similar symptoms were identified in France where a group was formed to study the new disease led by Dr. Montagnier [6]. The most obvious feature of the disease was immunodeficiency, in particular depletion of circulating CD4+ T cells [1, 2]. Initially some believed that the symptoms were related to behavior specific to gay men, which is the reason why it was termed "the gay disease" [7, 8]. However, this claim was quickly dismissed when AIDS was observed in intravenous drug users in the United States[8]. Researchers soon postulated that the cause of the syndrome was an infectious agent, most likely a virus that is transmitted through sexual contact and blood [6, 8, 9]. Nonetheless, the epidemiologic and clinical data could not be linked with any know pathogen. The latter prompted an extensive search for the novel human virus.

The discovery of interleukin-2 (IL-2) enabled T cell culturing. In turn, this allowed Dr. Gallo and colleagues to identify human T cell leukemia virus (HTLV- 1) and

subsequently HTLV-2 [10]. HTLVs are human retroviruses and were the only known human viruses which preferentially infected CD4+ T cells [8, 10]. Moreover, HTLV-1 is transmitted through sexual contact, blood and from mother to child in addition to causing a mild immunodeficiency[10]. Based on these findings, Gallo and colleagues speculated that the unknown agent causing immunodeficiency in humans was a retrovirus closely related to HTLV [7]. This hypothesis was only partially correct. Although the pathogen responsible for causing AIDS was a retrovirus, it was not a variant of HTLV [11].

Since the individuals affected by the novel disease had severely decreased levels of circulating CD4+ T cells, it seemed obvious that these cells were the major target of the pathogen and were being destroyed as a consequence of the viral cytopathicity. The apparent lymphadenopathy observed early in infection meant that there is a strong immune response to the virus. Therefore, the best approach to isolate the virus was to sample peripheral blood and lymph nodes of newly infected individuals[6]. In January 1983, Barre-Sinoussi and associates, isolated and cultured lymph node cells isolated from a biopsy sample of a patient suffering from generalized lymphadenopathy [6, 7, 11]. Cell culture supernatants were assayed for reverse transcriptase (RT) activity, and were found positive. Electron microscopy detected retrovirus-like particles budding from the plasma membrane. However, their morphology was different in comparison to HTLV. While immunofluorescence identified specific antibodies against the virus in sera of two patients, type-specific antisera to HTLV-1 were nonreactive [11]. The cumulative data clearly demonstrated that this was indeed a new human retrovirus. Initially it was named lymphadenopathy associated virus (LAV) or HTLV-3, but was later dubbed human immunodeficiency virus (HIV) [6, 10, 11]. Compiled data from both Dr. Montagnier's

and Dr. Gallo's laboratories would further link and reinforce the notion that HIV is the causative agent of AIDS [6, 10-12]. Soon after the discovery of HIV, another AIDS-causing human retrovirus related to HIV was isolated from two patients from West Africa [13]. This virus was first called LAV-2, but was renamed to HIV-2. Sequence analysis showed that this virus was evolutionarily distinct from HIV-1 [14].

HIV Epidemiology

AIDS has proven to be the defining medical and public health issue of the 20th and 21st century [8]. Since the identification of HIV as the causative agent of AIDS in 1983 until 2006, approximately 65 million people have been infected with this retrovirus [11, 12, 15]. Of those, *circa* 25 million people have died as a consequence of AIDS [15]. However, the remaining 38.6 million infected individuals are incognizant of their seropositivity. Just in 2005, the mortality due to AIDS was 2.8 million whereas the incidence of HIV infection was 4 million. Sub-Saharan Africa has the highest prevalence of HIV infection at around 25 million people, although declines in Kenya and Zimbabwe have been observed. Epidemiologically speaking, it is alarming that the incidence of HIV infection is on the rise in Eastern Europe and Central Asia.

HIV Classification

The hallmark of retroviruses is the viral enzyme reverse transcriptase (RT) that converts the RNA encoded genome into a double stranded cDNA copy that is subsequently inserted into the genome of the target cell [16-19]. There are many diverse members of this viral family which infect a variety of vertebrates [20-23]. Originally,

retroviruses were classified based on their morphology. However, since then the genera of the Retroviridae family have been reclassified based on whether they encode additional regulatory proteins in addition to the major gene products (Table 1).

Table 1. Classification of retroviruses and their cognate tRNA primer

Genus	Complexity	Members	tRNA primer
Alpharetrovirus	Simple	Avian Leukosis Virus (ALV)	Trp
		Rous sarcoma virus	Trp
Betaretrovirus	Simple	Mason-Pfizer monkey Virus (M-PMV)	Lys1,2
Gammaretrovirus	Simple	Murine leukemia virus (MuLV)	Pro
Deltaretrovirus	Complex	Human T-cell leukemia viruses (HTLV-1, -2)	Pro
Epsilonretrovirus	Complex	Walleye dermal sarcoma virus (WDSV)	His
Lentivirus	Complex	Human immunodeficiency viruses (HIV-1, -2)	Lys3
		Simian immunodeficiency viruses (SIV)	Lys3
		Equine infectious anemia virus (EIAV)	Lys3
		Feline immunodeficiency virus (FIV)	Lys3
		Carpine arthritis encephalitis virus (CAEV)	Lys1,2
		Visna maedi virus	Lys1,2
Spumavirus	Complex	Human foamy virus	Lys1, 2

Relationships among retroviruses can be based on the *gag* (group specific antigen), *pol* (polymerase) and *env* (envelope) gene sequence. Regardless of the method used, clustering of particular viruses within genera is similar. HIV belongs to the *Retroviridae* family, *lentivirus* genus (*lat. lentus=slow*).

HIV Origin

HIV shows a marked genetic diversity due to its high replication rate, the relatively error-prone nature of reverse transcriptase, and recombination [24, 25]. Based on viral sequences HIV-1 strains are divided into three groups termed M (main), O (outlier) and N (non-M, non-O). The first isolated HIV falls into the M group, which is

further subdivided into clades, termed subtypes A to K. These viruses are responsible for the majority of the AIDS cases today. O strains are very divergent and endemic to western equatorial Africa. N strains have been isolated in Cameroon and comprise a small number of viruses. HIV-2 is even more divergent and is therefore subdivided into groups A to H.

Direct epidemiologic evidence on the source and time of HIV emergence in the human population is not available. However, molecular epidemiology of primate lentiviruses has provided the most accurate means to answer these questions. Various simian immunodeficiency viruses (SIV) are lentiviruses which infect 36 different species of nonhuman primate species [26]. Isolation of an SIV isogenic with HIV-1 from a naturally infected Gabonese chimpanzee (SIVcpz) provided the first clue that HIV-1 might have arisen through a cross-species transmission [27]. Since then, extensive molecular phylogeny analyses have determined that an SIV strain infecting a specific chimpanzee subspecies *Pan Troglodytes Troglodytes* (SIVcpz*Ptt*) is the natural reservoir of HIV-1 group M and N [28]. A direct link between SIV and group O HIV-1 has not been established as of yet.

The vast data obtained from the study of SIV suggests that the introduction of this virus into the human population occurred at numerous and different time points [29]. SIVcpz*Ptt* crossed over at least three independent times, thus creating HIV-1 groups M, N and O.

HIV-2 was established in the human population via a cross-species transmission of SIV infecting sooty mangabeys (*Cercocebus atys*) (SIVsm) [29]. SIVsm cross-species transmission happened at an even higher frequency, since there are more groups of HIV-

2. Estimates place the last common ancestor of HIV-1 belonging to group M as early as 1931 and as late as 1941 [24].

Morphology of the HIV-1 Virion

The mature HIV virion is an enveloped virus 80 – 100 nm in diameter [20] (Figure 1). The viral envelope is derived from the cellular plasma membrane. Acquisition of the envelope occurs during virus particle budding from the surface of the infected cell. Embedded in the envelope are the trimeric viral spikes formed from the *env* gene glycoproteins [30]. The monomers consist of the surface domain (SU/gp120) tethered to the transmembrane domain (TM/gp41). The major *gag* encoded proteins form the structural components of the virion. They include membrane associated or matrix (MAp17), capsid (CAp24) and mature nucleocapsid (NCp7) proteins. MAp17 lines the inside surface of the envelope. The CAp24 protein forms the "bullet shaped" core of the mature HIV-1 virion. Housed within the core is the pseudodiploid RNA genome bound to NCp7. The reverse transcriptase heterodimer (RT) is also bound to the viral genomic RNA (vgRNA). Components of the HIV-1 core include integrase (INp32), protease (PR), various tRNA species, tRNA synthetases, some viral accessory proteins (Vif, Nef, and Vpr), as well as other viral and cellular proteins.

HIV-1 Viral Genomic RNA (vgRNA)

The HIV-1 genome is linear, positive sense RNA, approximately 9.2 Kb in length [23, 31-33]. A self complementary region near the 5' end of the RNA termed the dimer-

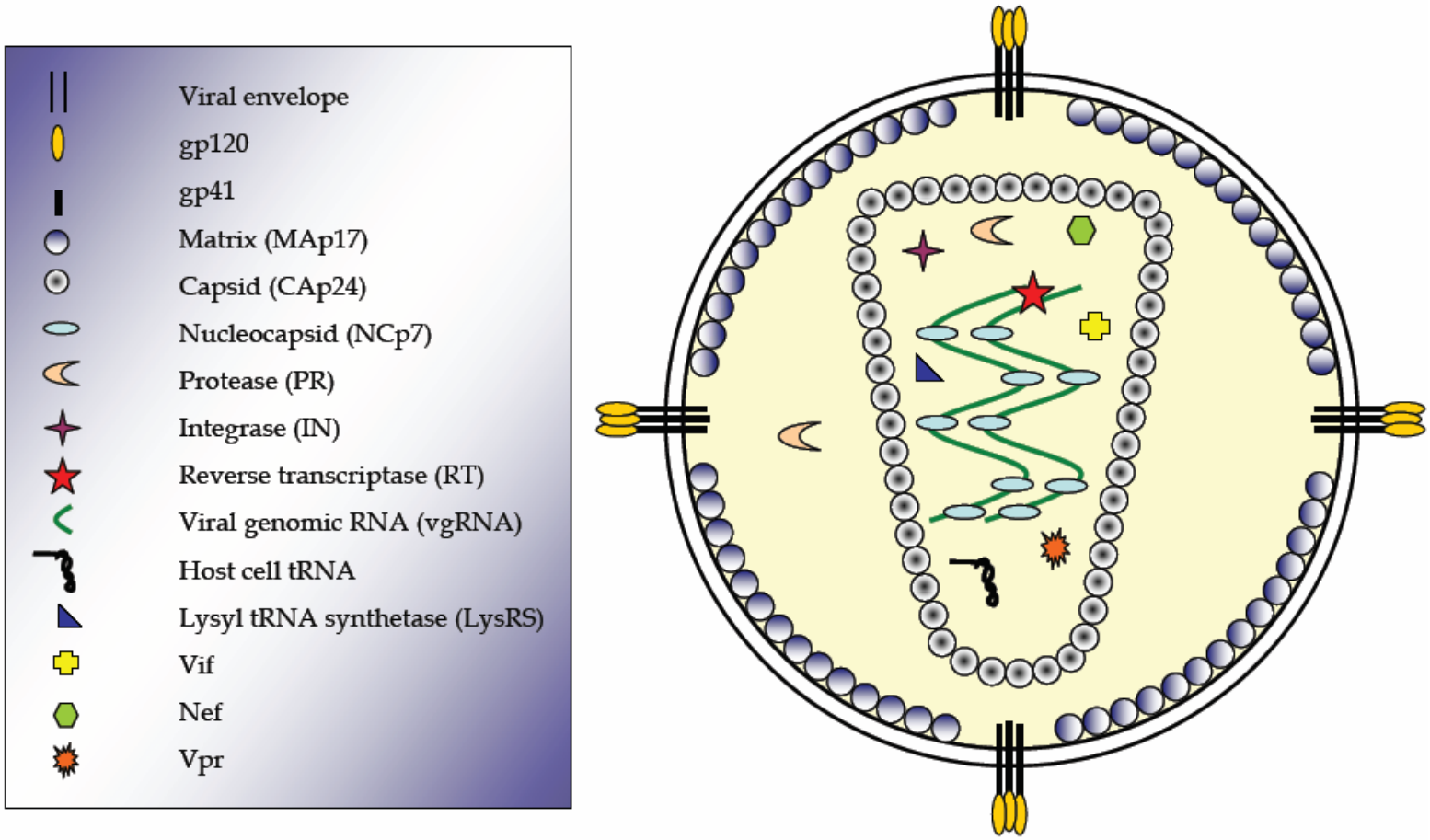

Figure 1. Morphology of the mature HIV-1 virion.
The mature HIV-1 virion is 80-100nm in diameter. gp120/gp41 trimers are embedded in the envelope and are used for virus attachment to the host cell. MAp17 is associated with the envelope. CAp24 proteins form a bullet shaped core which houses the NCp7-bound pseudodiploid linear vgRNA. The vgRNA is capped, polyadenylated and approximately 9.2 Kb in length. Virally encoded enzymes (PR, RT, and IN) are found the virion. Viral accessory proteins (Vif, Nef and Vpr) are packaged into mature HIV-1. Additionally, HIV-1 packages some host cell proteins, among which is LysRS as well as cellular various cellular tRNAs.

initiation site (DIS), tethers the two viral genomes encapsidated inside the virion [34]. The vgRNA contains the $_{m7}$G5'ppp5'G$_{m}$p cap at the 5' end of the transcript similar to all eukaryotic mRNA. Additionally, a polyadenosine (poly(A)) tail is located at the 3' end, approximately 200 nucleotides in length [30]. Although the HIV-1 vgRNA resembles eukaryotic messenger RNA (mRNA), it is not translated in the cytoplasm of the host cell following entry. Rather, it undergoes the process of reverse transcription which converts the vgRNA into a double stranded DNA (dsDNA) copy. Once integrated, the viral dsDNA is referred to as the provirus which serves as a template for transcription of viral RNA (vRNA). Following transcription, vRNA can be unspliced (usvRNA), singly spliced (ssvRNA) or multiply spliced (msvRNA). usvRNA is exported from the nucleus and serves not only as a template for translation of Gag and Gag-pol polyproteins (Pr55^{Gag} and Pr160$^{Gag\text{-}pol}$, respectively), but is encapsidated as vgRNA.

HIV-1 Genomic Organization

The genomic organization of HIV-1 is similar to other retroviruses [35] (Figure 2). The HIV-1 provirus has nine open reading frames (ORFs), which encode not only the major genes (*gag*, *pol,* and *env*) but also regulatory (*tat* and *rev*) and accessory genes (*vif, vpr, vpu,* and *nef*) which play important roles in infection *in vivo*. The provirus contains additional sequence (approximately 600 nucleotides in length) generated as a consequence of reverse transcription [18]. These sequences represent part of the terminal repeats (LTRs) which are located at both the 5' and 3' end of the integrated provirus [36-39]. *gag, pol, and env* are flanked by LTRs [36-39]. All nucleotide numbering cited in

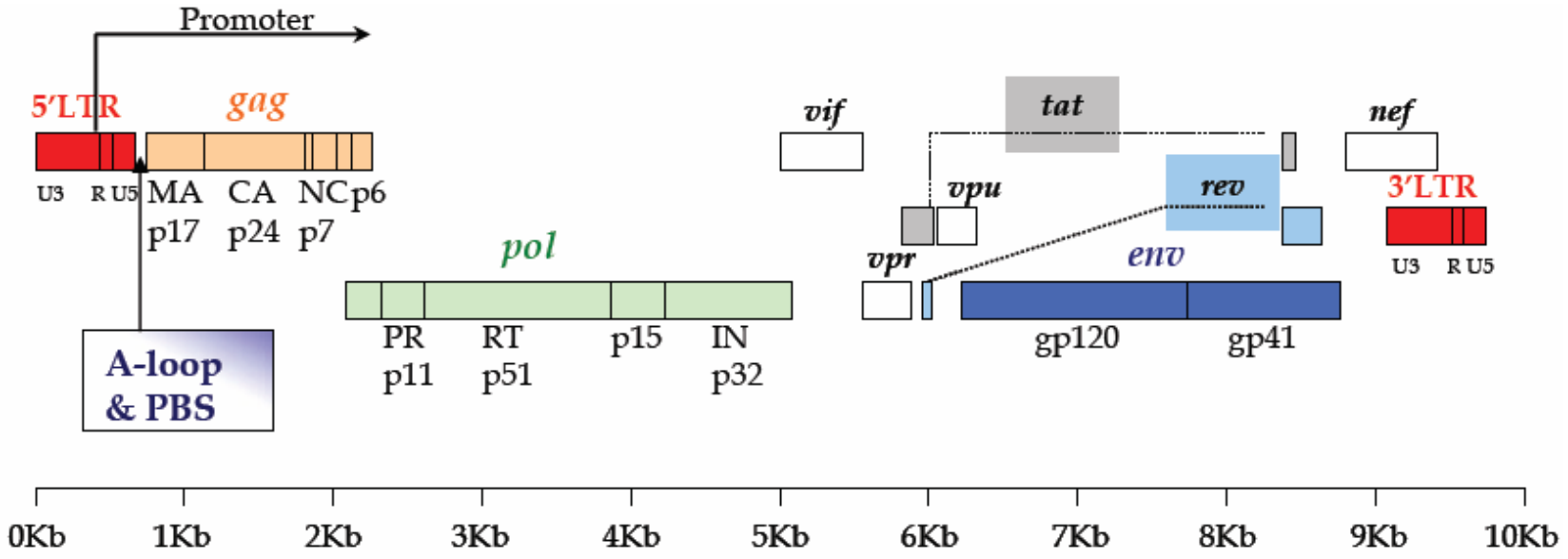

Figure 2. Genomic organization of the HIV-1 provirus.
Long terminal repeats (LTR) are identical noncoding sequences and contain transcriptional control and regulatory elements (red). They are located on both the 5' and 3' end of the provirus. Downstream of the 5' LTR is the primer binding site (PBS) which is complementary to the 18 3' terminal nucleotides of $tRNA^{Lys3}$ in wild type HIV-1 (partially shaded blue box). RT uses host cell tRNA bound to the PBS to initiate reverse transcription. There are three major genes in HIV-1-*gag* (orange), *pol* (pale green) and *env* (blue) which are translated as polyproteins ($Pr55^{Gag}$, $Pr160^{Gag\text{-}pol}$ and gp160, respectively). $Pr160^{Gag\text{-}pol}$ is the product of a frameshift event during translation of *gag* at the *gag-pol* frameshift site, resulting in translational recoding of the stop codon further downstream. $Pr55^{Gag}$ codes for MAp17, CAp24, NCp7 and p6. The pol portion of $Pr160^{Gag\text{-}pol}$ codes for the viral enzymes PR, RT and IN. Functional RT is a p66/p51 heterodimer (p51 is a result of PR cleavage of p66). gp160 is cleaved into gp120 (the surface portion) and gp41 (the transmembrane portion) of the HIV-1 spike. Regulatory proteins (Tat and Rev) are translated from *tat* (grey) and *rev* (pale blue), respectively. Accessory genes include *nef*, *vif*, *vpr*, and *vpu* (white boxes).

the text is based on the HXB2 clone sequence with the caveat that the RNA cap site is designated as +1 [35, 39].

Long Terminal Repeats (LTRs)

LTRs are identical noncoding sequences located at either end of the provirus [40]. They are 634 base pairs in length[35]. Going from 5' to 3', LTRs are further divided on the basis of function into three segments designated: unique 3' region (U3), repeat region (R) and unique 5' region (U5).

Unique 3' Region (U3). The U3 is a segment 454 nucleotides (nt) long and terminates at the transcription initiation site [35]. It contains promoter, enhancer and negative regulatory regions. The TATA box is located at -27 in relation to the RNA cap site designated +1 [41]. The TATA element is recognized and bound by the TATA-binding protein (TBP) portion of transcription factor II D (TFIID) [42]. TFIID is a member of the general transcription factors (GTFs) that aid in correctly positioning cellular DNA polymerase II at the transcription start site. Upstream of the TATA element (-46 to -77) are three consecutive elements (Sp1I to III) that bind Sp1, another cellular transcription factor [43]. The furthest upstream, Sp1III, has the greatest affinity for the transcription factor. The Sp1 and TATA elements form the HIV-1 core promoter [23]. They are required for LTR-driven transcription. The enhancer region is located upstream from the promoter region [23, 35]. It consists of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) binding sites which stimulate transcription *in vitro* [23, 44-46]. Of the three regions, the negative regulatory element (NRE) is the furthest

upstream [23, 35]. It contains binding sites for the upstream stimulatory factor (USF) and the E26 transforming specific (Ets) transcription factor [23].

Repeat Region (R). R is 96 nt long and starts at the transcription initiation site [35]. It contains a 5' untranslated leader sequence present in all HIV-1 mRNA. Initially, sequences identified as sufficient to respond to the virally encoded transactivator protein (Tat) were mapped to be located around the transcription initiation site (-17 to +80) [44, 47]. Therefore, this region was designated the transactivation response element (TAR) and is predominantly located in R. Further mutational analyses demonstrated that TAR encompasses the sequence from -17 to +44 and that its function is dependant on orientation and position [48-52]. However, subsequent experimental data have shown that Tat mediated transactivation occurs at the RNA level [35]. Specifically, following transcription, the first 59 nt of the vgRNA form a very stable RNA stem loop (designated TAR stem loop) [49, 52-54] (Figure 3). Virally encoded transactivator protein (Tat) binds TAR of vgRNA and stimulates transcription [44]. Therefore, the TAR stem loop is completely located in R (+1 to +59) [23, 53, 54]. The R region of the vgRNA also contains part of the poly(A) (pA) stem loop that contains the poly(A) signal. Mutations that disrupt base pairings of the TAR or pA stem structures severely impair packaging and viral replication [53]. Additionally, the U3-R junction is host to a sequence element that binds leader binding protein-1 (LBP-1) [23].

Unique 5' Region (U5). The U5 region is 84 nt in length [23, 35]. The poly(A) signal (ATAAA) is located on the pA stem loop, 19 nucleotides downstream of the R-U5

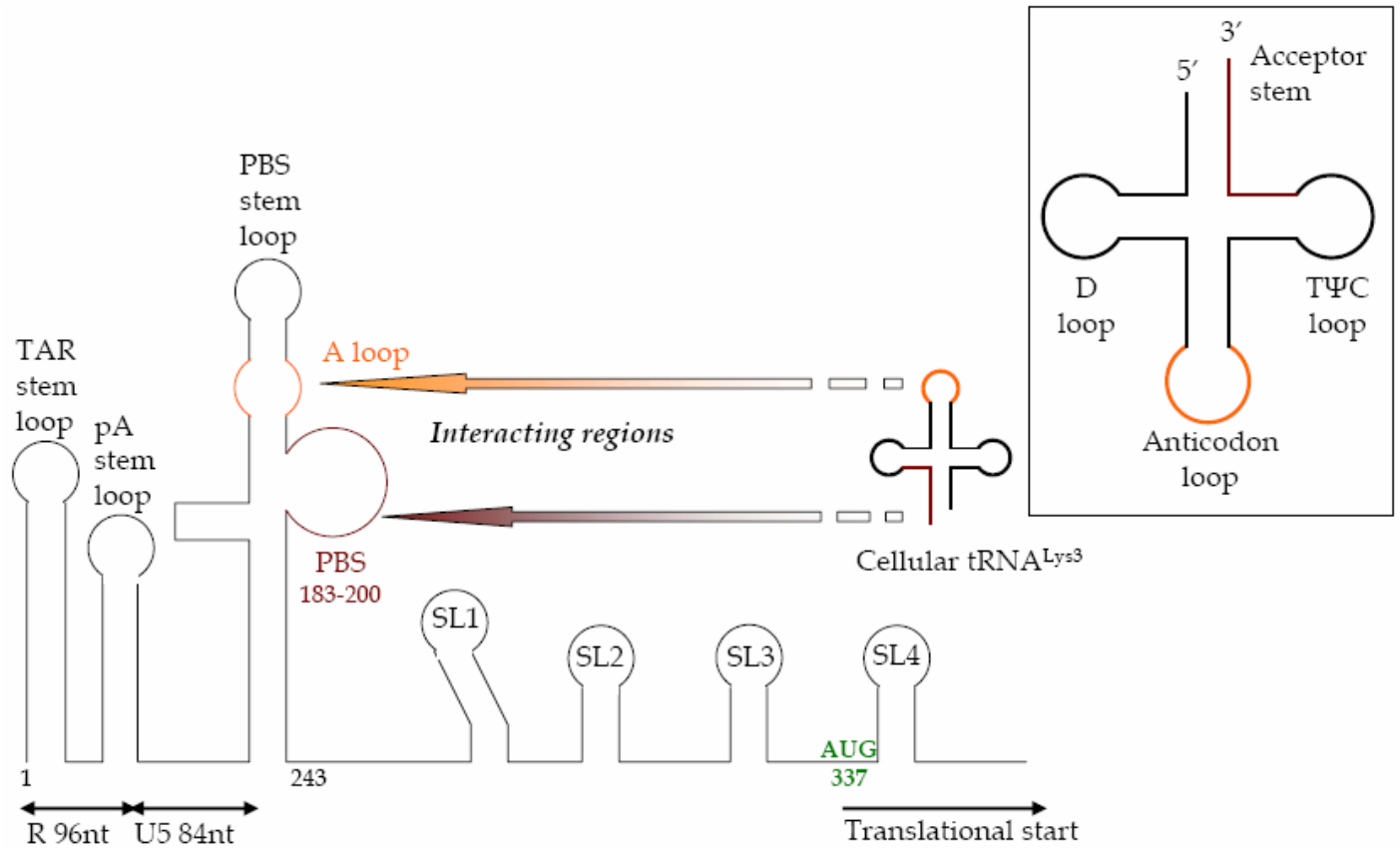

Figure 3. Predicted secondary folds of $tRNA^{Lys3}$ and the 5' end of HIV-1 viral genomic RNA (vgRNA).
Transcription of HIV-1 provirus starts at the 5' U3 R boundary. The 5' end of the viral genomic RNA contains many stem loops including the transactivation response element (TAR) stem loop, poly A (pA) stem loop, primer binding site (PBS) stem loop, and a series of stem loops (SL1, SL2, SL3 and SL4). The *gag* start codon (AUG) is indicated (dark red). The PBS stem loop contains the A-loop and the PBS. These are complementary, and interact with the anticodon and the 18 3' terminal nucleotides of $tRNA^{Lys3}$ in wild type HIV-1, respectively. The interaction is represented by green and red shaded block arrows. A larger representation of $tRNA^{Lys3}$ is shown in the upper right hand corner. The 18 3' terminal nucleotides are represented by a red line, while the anticodon loop is orange. The TΨC loop and the D loop are also shown.

junction. The region has homology to a motif called the G-T cluster that is important for efficient polyadenylation of cellular RNA and vRNA[55]. vRNA is polyadenylated from the 3' LTR polyadenylation signal.

Important Sequences Downstream of the HIV-1 LTR

Immediately following the 5' LTR are the adenosine-rich (A-loop) and primer binding site (PBS) sequences [23, 35, 56]. In transcribed vgRNA, the A-loop and PBS are located on the PBS stem loop. Both of these sequences interact with cellular $tRNA^{Lys3}$ in wild type HIV-1 [21, 23, 57-59]. The PBS is complementary to the 3' 18 terminal nucleotides of $tRNA^{Lys3}$. Initiation of reverse transcription requires $tRNA^{Lys3}$ to anneal to the PBS. The A-loop interacts with the anticodon loop of $tRNA^{Lys3}$ [57]. Sequences downstream of the PBS stem loop are predicted to form a series of four stem loop structures (SL1-4) [60]. SL1, SL2, SL3 and SL4 have overlapping functions and contain at least one of the HIV-1 vgRNA packaging signals (Ψ). Moreover, SL1 contains the primary DIS, while SL2 contains the major splice donor site [34, 60].

Major Viral Proteins

Major viral proteins are translated as polyprotein precursors from usvRNA ($Pr55^{Gag}$ and $Pr160^{Gag\text{-}pol}$) and ssvRNA (gp120) [21, 23, 58]. Following release of viral particles PR cleaves the polyproteins.

Gag Polyprotein (Pr55^{Gag})

The first of the nine ORFs present in HIV-1 usvRNA, encodes the Gag polyprotein (Pr55^{Gag}) [61]. Pr55^{Gag} is a 55 kDa precursor of the HIV-1 structural proteins (MAp17, CAp24, and NCp7), and a protein responsible for virus particle release (p6). Synthesis of Pr55^{Gag} is initiated at the ATG located at nucleotide 334 and terminated at nucleotide 1837 [35]. However, it is important to note that in addition to the canonical cap-dependent mode of translation, some data suggest that an internal ribosome entry site (IRES) is present near the *gag* ORF [56]. Although controversial, this would allow Pr55^{Gag} to be translated via a cap-independent mechanism. The significance of this data has to be viewed in the context of the long 5' untranslated region (5'UTR) of the usvRNA. The 5' UTR not only forms stable RNA stem loops that would inhibit scanning of the initiation complex, but also contains AUG codons in a favorable translation initiation context that would result in synthesis of an out of frame Pr55^{Gag}. However, apparent conundrum could be potentially explained by the process of "leaky scanning" [42]. AUG codons can be skipped during translation initiation as a mechanism of translation regulation. Translation will become processive only when the interaction of the initiation complex and the AUG in the proper context is strong enough. Although the first encountered AUG typically marks translation initiation, it is not always the case.

Pr55^{Gag} orchestrates virus assembly at the cellular plasma membrane. Following release of viral particles, Pr55^{Gag} is cleaved by PR into MAp17, CAp24, and NCp15. These events dramatically alter the structure of the immature virus particle in a process termed maturation.

Matrix domain(MA) and MAp17. In the context of $Pr55^{Gag}$, MA is located at the amino terminus. $Pr55^{Gag}$ is cotranslationally modified by the addition of a myristic acid to a glycine residue in MA [23]. This reaction is catalyzed by myristyl CoA transferase. In addition to the hydrophobic residues found in the MA domain of the polyprotein, myristoylation aids $Pr55^{Gag}$ localization and association with the cellular plasma membrane. Within the mature virion, MAp17 is bound to the viral envelope. During maturation, viral PR excises MAp17 from $Pr55^{Gag}$. Although MAp17 associates with lipid bilayers, its relative binding affinity is lower in comparison to $Pr55^{Gag}$. The cause of the decreased affinity was attributed to the "myristyl switch", an event that results in masking the myristic acid posttranscriptional modification on MA. Initially it was postulated that $Pr55^{Gag}$ processing was the cause of the myristyl switch. However, subsequent experiments determined that $Pr55^{Gag}$ oligomerization causes the myristate to be exposed thereby enhancing binding affinity towards lipid bilayers. Therefore, sequestration of the myristate is not the result of $Pr55^{Gag}$ processing *per se*, rather it is the effect of $Pr55^{Gag}$ oligomer dissociation during PR cleavage of the polyprotein during maturation.

MA also plays a role in the incorporation of viral Env glycoproteins into virions. Finally, as a component of the preintegration complex (PIC), MAp17 has been implicated in aiding nuclear import of this structure through its nuclear localization sequence (NLS) [62, 63].

Capsid Domain(CA) and CAp24. CA can be structurally and functionally divided into the N-terminal domain (NTD) and the C-terminal domain (CTD) [23]. These two domains are connected by a short linker.

Mutational analyses have shown that $Pr55^{Gag}$ multimerization is accomplished via CA:CA interaction [64]. The CA CTD mediates this interaction. Since $Pr55^{Gag}$ oligomerization is essential for virion assembly, the CTD is crucial for HIV-1 viability. Additionally, The CTD contains a 20 nt sequence called the major homology region (MHR) which is highly conserved among retroviral CA proteins [65]. The MHR is essential for viral assembly, maturation and infectivity. However, crystal structures reveal that the MHR is distinct from the dimer interface. Rather it forms a complex hydrogen bonding network with other regions of CA. During maturation, PR cleaves $Pr55^{Gag}$ to produce CAp24. Purified CAp24 is a 24 kDa protein that will self-assemble into tubular structures *in vitro* [61, 66]. In mature HIV-1, these proteins remain tethered to each other and form the HIV-1 core. CAp24 can be detected by using an ELISA which contains antibodies directed against the protein. An increase of CAp24 in culture supernatant is indicative of a productive infection.

During assembly, the NTD interacts with cellular cyclophylin A (Cyp A) which physiologically functions as a peptidyl-prolyl cis-trans isomerase [67]. This binding event results in Cyp A incorporation into virions [68, 69]. The significance of this interaction is still not fully understood. However, it seems that the CypA binding to CA in the target cell, but not the producer cell is important in regulating virus infectivity [70].

Nucleocapsid Domain (NC) and NCp7. NC represents the third major domain of the $Pr55^{Gag}$ [23]. It contains two clusters of basic residues that flank the first of the two zinc-finger motifs (of the CCHC type=Cys-X2-Cys-X4-His-X4-Cys) present in the protein [71]. These features are indicative of nucleic acid binding proteins. The primary role of NC is to sequester usvRNA into the virion by interacting with Ψ (located in the 5' UTR) [60].

In addition to its primary role, NC exhibits many other functions during the HIV-1 life cycle. Many HIV-1 NC mutants show defects in assembly and budding [23]. Therefore, NC has been implicated in $Pr55^{Gag}$ multimerization during assembly. The model predicts that NC-bound usvRNA provides an organizational template for assembly of additional $Pr55^{Gag}$.

PR cleavage of $Pr55^{Gag}$ results in the formation of the nucleocapsid precursor (NCp15). This protein is further processed in the virion to produce mature nucleocapsid protein (NCp7), p1, p2 and p6. NCp7 binds vgRNA in the core and catalyze its refolding. It has been postulated that NCp7 protein binding to vgRNA converts the kissing dimer to a more thermodynamically favorable duplex [60].

NC and/or NCp7 are thought to have a role during virtually every step of reverse transcription: placement of the tRNA primer, unwinding of the tRNA primer during initiation, promoting both strand transfer events, removing annealed RNA during negative strand synthesis, and destabilization of RNA structures during plus and minus strand synthesis [23].

p6 Domain and p6. The p6 domain is a proline-rich region, located at the $Pr55^{Gag}$ CTD [23]. It is characterized by the highly conserved Pro-Thr-Ala-Pro (PTAP) motif located near its NTD. The PTAP is required for proper virus release during budding [72]. Additionally, the p6 domain of $Pr55^{Gag}$ is responsible for Vpr incorporation into virions [73]. Following PR mediated cleavage of NCp15, the 6kDa p6 is released.

Gag-pol Polyprotein ($Pr160^{Gag-pol}$)

In the case of HIV-1, the second ORF overlaps the *gag* reading frame by 241 nucleotides [35]. *Pol* is translated as a consequence of ribosomal slippage by one nucleotide which creates a -1 ribosomal frameshift at a UUA leucine codon, resulting in translational recoding of the stop codon and formation of the 90-92 kDa $Pr160^{Gag-pol}$ [74]. A secondary RNA structure at the *gag-pol* junction stalls translating ribosomes. As a result, ribosomes frameshift (10% frequency) at the slippery sequence consisting of 6 uracils located just upstream of the RNA hairpin [35, 74, 75]. Frameshift efficiency is a crucial determinant of virus viability, since relative proportion of $Pr55^{Gag}$ to $Pr160^{Gag-pol}$ influence viral assembly [56]. Expression of only $Pr160^{Gag-pol}$ seems to abrogate virion assembly. The Pol portion of the Gag-pol fusion protein codes for the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN). These enzymes are cleaved from $Pr160^{Gag-pol}$ during maturation of virions. Incorporation of $Pr160^{Gag-pol}$ into the immature virion is mediated through interaction of $Pr55^{Gag}$ and $Pr160^{Gag-pol}$ CA domains.

Protease (PR) and PRp11. HIV-1 PR is an aspartic protease crucial to virus viability, since it catalyzes cleavage of the $Pr55^{Gag}$ at two, and $Pr160^{Gag-Pol}$ at five sites

[76]. PRp11 is autocatalytically cleaved from the $Pr160^{Gag\text{-}pol}$ during maturation of HIV-1 [77]. Functionally active PR is a homodimer of PRp11. It has sequence and structural similarities to cellular aspartic proteases of the pepsin family [78]. The active site of the enzyme is formed at the interface of the two monomers, each containing a catalytic triad (Asp-Thr-Gly).

Initial products of $Pr55^{Gag}$ cleavage are MAp17, CAp24, and NCp15 [58]. In addition to MAp17, CAp24, and NCp15, $Pr160^{Gag\text{-}pol}$ cleavage results in release of PRp11, RTp66 and INp32. Secondary cleavage of NCp15 results in production of NCp7, p1, p2, and p6. RTp66 is also further cleaved to produce RTp51 and p15.

Reverse Transcriptase (RT) and RTp66. The process in which the vgRNA is converted to a dsDNA copy is mediated by the virally encoded reverse transcriptase enzyme [16, 18, 19]. Functional RT is a heterodimer formed by the association of RTp66 and RTp51 monomers [79]. Initially, the RTp66 monomer is cleaved from $Pr160^{Gag\text{-}pol}$ by viral PR [76]. Further processing of the p66 subunit by PR results in the RNaseH deficient p51 subunit and an inactive p15 RNaseH product [59]. HIV RT has been crystallized either complexed with nevirapine or dsDNA [80-82]. The crystal structure of RTp66 resembles a human hand grasping the primer template complex [58]. Structurally, it can be divided into the following five subdomains: Fingers, palm, thumb, RNaseH and connection. The polymerization site is localized to the palm subdomain, while tRNA primer recognition might occur through the thumb subdomain. The crystal structure of the RT complexed with dsDNA revealed that the heterodimer has an 18 nucleotide footprint. The 3' end of the 18 nucleotide dsDNA is located at the active site while the

5' end is situated near the RNaseH subdomain [80]. This complex supports previous findings that the 3' 18 nucleotides of tRNAs are used as primers for initiation of reverse transcription [17, 18, 83].

Functionally, RT is a very versatile and flexible enzyme since it has to accommodate the necessity for many different functions. Not only is it a RNA dependent DNA polymerase (RDDP), but also a DNA dependent DNA polymerase (DDDP). Additionally, it has DNA-RNA duplex dependent ribonuclease activity (RNase H) as well as RNA-RNA dependent ribonuclease activity (RNaseD) [59]. A schematic representation of an accepted model of reverse transcription is presented in Figure 4.

Integrase (IN) and INp32. INp32 is a 32 kDa protein which is proteolytically cleaved from $Pr160^{gag\text{-}pol}$ [23]. In the context of $Pr160^{Gag\text{-}pol}$, it is located at the CTD. The major function of IN is integration of the viral dsDNA into the host genomic DNA. Retroviral INp32 can be subdivided into three functionally and structurally distinct domains: The NTD, central core domain (CCD), and CTD. The IN NTD domain contains a highly conserved HHCC motif that is responsible for zinc cation binding [84-86]. Additionally, the NTD plays an important role in INp32 multimerization, since mutations or antibody blocking of this region abrogate multimer formation [86, 87]. Proper IN function requires both INp32 multimerization and the presence of divalent zinc ions. The CCD is responsible for catalysis although it has also been implicated in aiding multimerization [84, 88]. Although the IN CTD exhibits potent nonspecific DNA binding properties, it is unknown what function it serves.

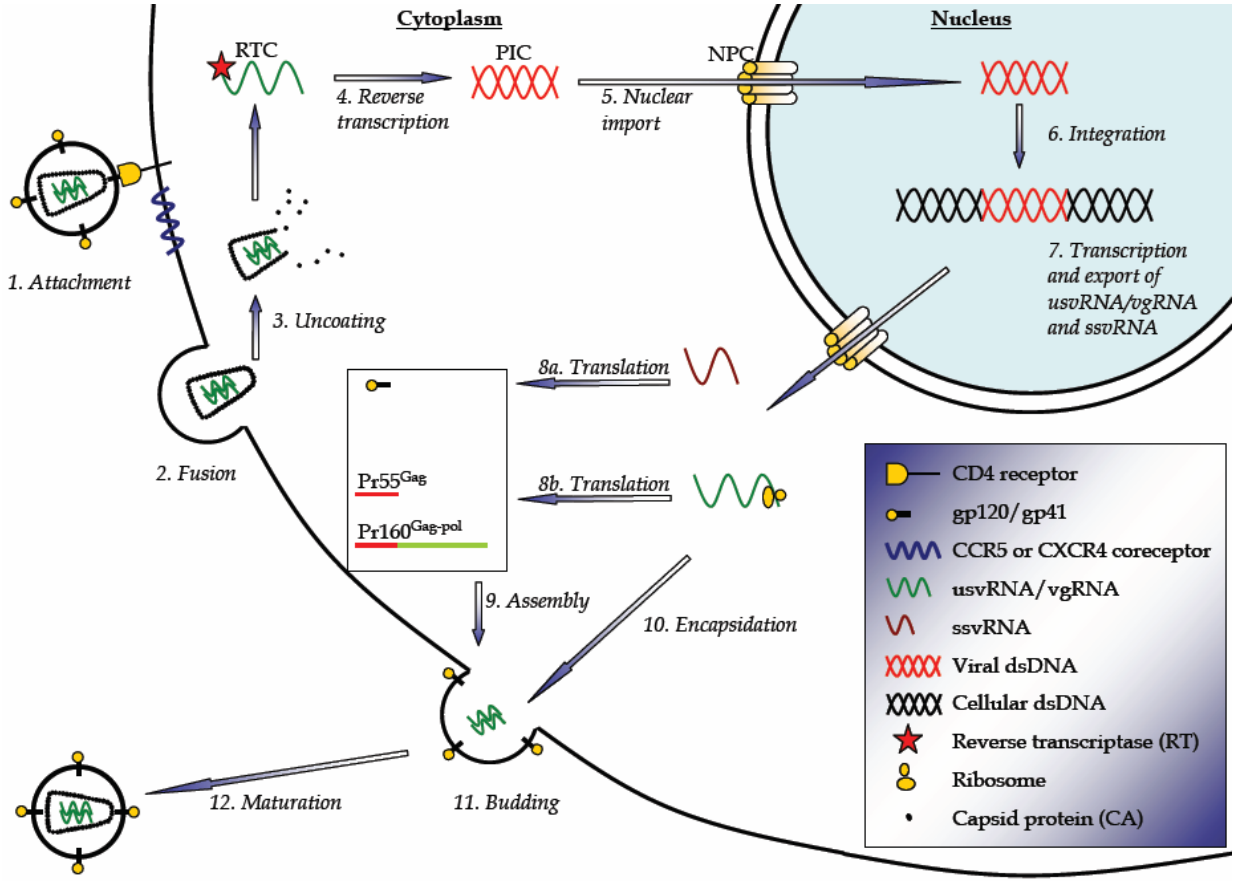

Figure 4. HIV-1 replication cycle.
HIV-1 binds to a target cell by gp120 interaction with a CD4 receptor and a CCR5 or CXCR4 coreceptor. Following fusion of the viral envelope with the cellular plasma membrane, the viral core is inserted into the cytoplasm, where it begins to disassemble. Mediated by RT, the viral genomic RNA (vgRNA) in the reverse transcription complex (RTC) is converted to a dsDNA copy. The RTC becomes the preintegration complex (PIC) which is imported through the nuclear pore complex (NPC) into the nucleus. Viral dsDNA integrates into the host cell genome. The provirus serves as a template for transcription of viral RNA. Following transcription, the unspliced viral RNA (usvRNA) is exported through the NPC into the cytoplasm of the host cell where it acts both as a template for $Pr55^{Gag}$ and $Pr160^{Gag\text{-}pol}$ synthesis and as vgRNA which is to be packaged into budding virions. Following assembly, the HIV-1 particles bud from the cellular plasma membrane. PR-mediated cleavage is termed maturation.

Env Precursor (gp160)

The *env* gene ORF stretches from nucleotides 5781 to 8369, with the ATG located at nucleotide 5802 [39]. The translated product is the Env precursor polyprotein (gp160) containing approximately 850 amino acids [89]. *env* mRNA is a singly spliced bicistronic message obtained from joining the 5' leader sequence to the coding portion of the *env* gene. The splice donor is located at nucleotide 287, whereas the splice acceptor site is either at nucleotide 5358 or 5557 [37, 90]. Translation of this ssvRNA occurs at the rough endoplasmatic reticulum (rER) [23, 30]. gp160 is glycosylated, folded and oligomerized at the rER. Typically, gp160 molecules form trimers. These modifications are important, since many *env* mutants show defects in oligomerization. Following translation, the polyprotein is transported to the Golgi apparatus where cellular furin proteases endoproteolytically cleave the polyprotein into two distinct glycoproteins-SU/gp120 and TM/gp41. Prior to a yet undefined transport mechanism to the cellular plasma membrane, gp120 and gp41 associate via weak noncovalent disulfide bonds. During transport, the whole complex (in particular gp120) is heavily glycosylated. These oligosaccharide modifications account for approximately 50% of the complex's molecular weight. Once at the plasma membrane, the TM portion of the trimers is inserted into the plasma membrane.

Surface glycoprotein (SU/gp120). As it name implies, SU is located at the surface of HIV-1 envelope[22]. Its major function is to interact with the CD4 receptor and CCR5 or CXCR4 coreceptor of the target cell. Heavy glycosylation of gp120 is thought to act as a shield, preventing host immune recognition.

Transmembrane glycoprotein (TM/gp41). TM is a single-pass transmembrane anchor. It spans the lipid bilayer of the plasma membrane through its Tyr-X-X-Leu domain [22]. Some studies suggest that this motif is also responsible for rapid internalization of the gp120-gp41 complex at the plasma membrane. The major role of TM is fusion of the viral envelope with the target cell plasma membrane in infection. Fusion is mediated through the hydrophobic fusion peptide present in TM.

HIV-1 Regulatory Proteins

Most genetic regulatory mechanisms consist of two components. Typically a protein acts as an effector molecule that binds a *cis*-acting responsive element which is usually a nucleic acid sequence [61]. HIV-1 encodes two regulatory genes that accelerate virus replication. *tat* and *rev*, code for trans-activator (Tat) and regulator of virion protein expression (Rev), respectively. These two proteins represent diffusible effector molecules that bind secondary vRNA structures directly and regulate transcription. Both proteins are translated from msvRNA[21].

Trans-activator (Tat)

The *tat* gene increases vRNA production through a positive feedback mechanism [47]. Although Tat is not packaged into virions, *tat* is an essential gene, since Δ*tat* HIV-1 mutants have been shown neither to replicate nor produce progeny virions [91, 92]. HIV-1 Tat is a nuclear protein found in two forms in the cell. *tat* mRNA is obtained when an exon located in the middle of the central region of the genome is spliced to a 3' splice acceptor [61]. The longer Tat protein is encoded by two exons and contains 101 amino

acids [23]. The shorter 72 amino acid Tat is encoded by one exon only. This form of Tat is the result of translational halting due to the termination codon located between the two exons. In tissue culture infections and LTR-driven reporter gene experiments, both forms of Tat exhibit transcriptional activation properties. The termination codon following the first exon of Tat is highly conserved among various HIV-1 isolates, suggesting that both forms of the protein might mediate different functions in an *in vivo* infection.

Tat promotes increased translation initiation and processivity through its interaction with the transactivation response element (TAR). The TAR stem loop consists of the first 59 nucleotides of the 5' terminal end of all viral mRNAs [52-54]. It forms a stable RNA stem loop structure [49, 52]. The three critical components of TAR include a base paired stem, a trinucleotide bulge (UCU starting at position +23), and a hexanucleotide G-rich bulge [93, 94].

Promoter elements present in the HIV-1 5' LTR (TATA, Sp1, AP-1), aid in both positioning the cellular RNA polymerase II (RNAPII) at the transcription initiation site and assembly of the preinitiation complex. Even though transcription initiates, elongation is very inefficient [95]. Nonetheless, RNAPII synthesizes very short nonpolyadenylated transcripts. The transcripts contain the TAR sequence whose folding induces formation of the TAR stem loop. Once translated, Tat localizes to the nucleus of the infected cell. There it associates with the human positive-transcriptional elongation factor (P-TEFb) which is formed by the association of cyclin T1 with Cdk9 [96]. Specifically, Tat interacts with the cyclin T1 component of P-TEFb [95]. The binding event triggers a conformational change in Tat which increases its affinity for association with TAR of the short transcript. The newly formed complex between Tat and P-TEFb

allows the Tat and cyclin T1 arginine-rich motifs (ARM) to bind the 5' bulge region and central loop of TAR, respectively. This binding event positions Cdk9 proximally to RNAPII. Once in position, Cdk9 hyperphosphorylates the RNAPII C-terminal domain, thereby inducing transcription elongation.

Regulator of Virion Protein Expression (Rev)

Eukaryotic cells have evolved many post-transcriptional mechanisms (5' capping, 3' polyadenylation and splicing) which ensure that the mRNAs that are transported to the cytoplasm code for functional proteins [42]. However, HIV-1 requires the export of usvRNA and ssvRNA for expression of the major gene products (Gag, Pol, and Env). In order to overcome this problem, HIV-1 has evolved a mechanism by which an effector protein binds an RNA structure present in usvRNA and ssvRNA and mediates their export to the cytoplasm [61].

The regulator of virion protein expression (Rev) is a 19 kDa protein containing 116 amino acids [23, 95]. Rev contains two exons and is expressed from msvRNA early in replication. It contains an arginine-rich nuclear localization sequence (NLS) as well as a leucine-rich nuclear-export sequence (NES). In addition to acting as a NLS, the arginine-rich domain also utilized for binding RNA. Sequences flanking the arginine-rich domain are responsible for multimerization of Rev. The major responsibility of Rev is to regulate expression and utilization of vRNA.

Following translation in the cytoplasm, Rev is transported to the nucleus. Nuclear transport is mediated through the interaction of the Rev NLS and importin-β. Importin-β belongs to a family of transport receptors which mediate nuclear import through the

nuclear pore complex (NPC). The NPC itself is comprised of nucleoporins [42]. Sequential association and dissociation of importin-β to a specific subset of nucleoporins translocate Rev into the nucleus. It is important to note that cellular proteins carrying a NLS usually first bind to importin-α which in turn binds to importin-β, bridging the cargo with the transport receptor.

Once in the nucleus, Rev associates with the *cis*-acting Rev response element (RRE). The latter is a complex RNA stem loop structure located in the *env* gene of all usvRNA and ssvRNA. Rev has a high affinity for binding a small portion of the RRE, designated the IIB stem loop. This initial binding event triggers multimerization of Rev on the remainder of the RRE. Following multimerization, Rev interacts with chromosome region maintenance 1 (Crm1) also known as exportin 1 protein in the presence of RanGTP. Ran switches between GTP and GDP bound states and transports the complex to the NPC, where exportin 1 mediates export of Rev-bound vRNA.

HIV-1 Accessory Proteins

There are four HIV-1 accessory proteins-negative factor (Nef), virion infectivity factor (Vif), viral protein r (Vpr) and viral protein u (Vpu). These proteins are encoded by *nef*, *vif*, *vpr* and *vpu*, respectively. Whereas Vif, Vpr and Vpu are translated from ssvRNA, Nef is translated from msvRNA.

Negative Factor (Nef)

The *nef* ORF is located at the 3' end of *env* and extends into the 3' LTR [35]. Nef is a 27 kDa protein, translated from msvRNA [23]. It is expressed early in infection and

can be found both in the cell and the virion. Within the virion, it associates with the viral core. Following translation, Nef is both myristoylated and phosphorylated. These post-translational modifications are crucial for Nef functionality. In the context of the infected cell, Nef interacts with the plasma membrane via the covalently linked myristoyl residue and a cluster of N-terminal basic residues [97].

Nef coordinates many events that seem to create an optimal environment for viral replication in the infected cell. Its major function is to downregulate both CD4 and major histocompatibility class I (MHC-I) molecules on the surface of infected cells [98-100]. Nef binds to the cytoplasmic domain of CD4 resulting in a cascade of events that lead to endocytosis of CD4 molecules. Internalized CD4 molecules are targeted to the cellular lysosomal pathway and subsequently degraded. The decreased amount of CD4 molecules on the surface of infected cells presumably lowers the possibility of a disadvantageous superinfection. Additionally, Nef downregulates MHC-I expression on the cell surface by redirecting the trafficking of newly synthesized MHC-I molecules from the trans-Golgi to the cellular endocytic pathway [101]. Although downregulation of MHC-I involves the cellular endocytic pathway, the mechanism is distinctly different from CD4 internalization. Reduced expression of MHC-I on the cell surface reduces antigen presentation of virally infected cells thereby reducing CD8+ cytotoxic T lymphocyte (CTL) mediated lysis.

Nef indirectly increases virus production. It prolongs the life span of an infected cell by countering cellular apoptotic mechanisms [95]. Moreover, Nef binds p53 thereby inhibiting its function in apoptosis. Finally, Nef increases viral infectivity and T-cell activation, although the exact mechanism has yet to be elucidated.

Virion Infectivity Factor (Vif)

Vif is a 23 kDa protein that is translated from a ssvRNA and packaged in the virion [35]. The *vif* gene is conserved in all lentiviruses, with the exception of EIAV [23]. Studies involving *vif* defective mutants demonstrated that its necessity is producer-cell dependent. Viral replication was supported in many (permissive) but not all (nonpermissive) cells. These findings suggested that Vif function is dependant on a specific cellular factor that is not necessarily expressed by all cells. Permissive and nonpermissive cell fusion experiments indicated the presence of a cell-specific dominant inhibitory factor, which was later identified and isolated [102-104]. The factor is now known as apolipoprotein B mRNA-editing enzyme catalytic polypeptidelike 3G (APOBEC3G). It is a member of cytosine deaminase proteins which are involved in mRNA editing [23]. In the absence of Vif in APOBEC3G expressing producer cells, this protein is packaged into the virions. Following infection of new target cells, APOBEC3G converts the cytosines to uracils during minus strong stop DNA synthesis. Presumably, cytosine deamination during reverse transcription results in G to A hypermutation of the viral RNA thereby triggering host mediated degradation of the reverse transcribed DNA.

Viral Protein r (Vpr)

The Vpr protein is a 14 kDa protein, expressed from ssvRNA [105]. It is efficiently incorporated into budding virions at high levels [106]. Vpr incorporation is accomplished through its interaction with the leucine-rich region of the $Pr55^{Gag}$ p6

domain [107, 108]. This HIV-1 accessory protein has been implicated in many stages of the viral life cycle. It is a major component of the preintegration complex and can be found in the nucleus. Vpr seems to participate in active transport of the preintegration complex (PIC) into the nucleus of non-dividing cells [109]. Additionally, it arrests the cell cycle at the G2/M phase while transactivating expression from the viral LTR [48].

Viral Protein u (Vpu)

Vpu is an HIV-1-specific (81 amino acids in length) protein that is expressed late in the viral life cycle from a *vpu-env* bicistronic vRNA at levels comparable to $Pr55^{Gag}$ [23]. It multimerizes and associates with the plasma membrane. In an infected cell, Vpu has two major functions: It enhances particle assembly/release and rapidly degrades CD4 molecules [110-112]. Although it is still unknown how Vpu enhances functional particle release, mutational analyses of *vpu* deficient HIV-1 clones reveal intracellular accumulation of viral proteins accompanied by a decreased amount of virus in the supernatant [110]. On the other hand, the mechanism of Vpu mediated CD4 degradation has been largely elucidated. The association of viral gp160 with CD4 in the endoplasmatic reticulum (ER) traps both proteins in this subcellular compartment [111]. Vpu degrades the CD4 molecule of the complex facilitating the release of gp160. In addition to liberating the Env precursor protein, Vpu aids Nef in downmodulating CD4 expression on the cell surface [112].

HIV-1 Replication Cycle

HIV-1 replication cycle can be subdivided into early and late events (Figure 4). Early events include virus binding, entry, uncoating, reverse transcription, nuclear import and integration. Late events include viral gene expression, assembly, budding, release and maturation.

Binding and Entry

The primary receptor required for HIV binding is CD4. CD4, a 55 kDa protein, is a member of the immunoglobulin (Ig) superfamily [23]. Its physiologic function is the stabilization of T cell receptors on T lymphocytes with major histocompatibility complex type II (MHC-II) molecules of antigen presenting cells (APCs). Although CD4 is the main receptor used by HIV, it was soon discovered that its presence on target cells is not sufficient for successful fusion of the viral envelope with the cell membrane. A coreceptor is also required for membrane fusion and virus entry. CCR5 and CXCR4 are seven transmembrane domain receptors that normally bind CC chemokines [25]. However, in HIV-1 infection, these receptors have been identified as the major coreceptor used by the virus to gain entry into the cell. CCR5 is predominantly expressed on dendritic cells, macrophages and CD4+ T cells, while CXCR4 is expressed on activated T cells.

HIV tropism is defined by the coreceptor it uses to gain entry into cells [25]. R5 isolates use CCR5 and were previously termed M-tropic, since they infect macrophages but not T cell lines *in vitro*. X4 isolates use CXCR4 and infect CD4+ T cells *in vivo*, and were therefore called lymphocyte tropic. Dual-tropic HIV-1 strains can use either CCR5

or CXCR4 as the coreceptor, and are designated R5/X4 strains [23]. R5 are the main transmitted strain, while X4 viruses emerge later in infection and are indicative of rapid progression to AIDS [113]. Further proof that R5 are the primary transmitted strain lies in studies with individuals who are only rarely infected by HIV-1, and are homozygous for a 32 base pair deletion in CCR5. The deletion encodes a truncated CCR5 which is poorly expressed on cell surfaces and cannot be used as a coreceptor in HIV infection [23]. Even though HIV/SIV can utilize many other chemokine receptors and related proteins to induce fusion *in vitro*, it is unclear whether their *in vivo* function is equivalent.

Upon gp120 binding to CD4 of the host cell, conformational changes of both molecules allow gp120 to interact with the CCR5 or CXCR4 coreceptor. Following this interaction, the gp41 NTD (termed fusion peptide) is inserted into the target cell lipid bilayer. Conformational changes in gp41 prompt formation of a six-helix bundle structure which induces membrane fusion. Following membrane fusion, the viral core is inserted into the cytoplasm of the host cell.

Uncoating

Viral core disassembly initiates as soon as it enters the target cell. This process is still poorly understood. However, recent data have shown that 70 % of capsid molecules in most mature infectious virions are not associated with the core [63]. Instead, they are free within the virus creating a metastable core via weak CAp24/CAp24 interactions. Hence, upon core release into the cytoplasm, these weak associations can be easily broken. The product of viral uncoating is the formation of the reverse transcription complex (RTC), which becomes the preintegration complex (PIC) once the process of

reverse transcription of the viral RNA is complete. While the exact composition of the RTC and PIC is still debated, data demonstrates that the PIC contains PR, RT, IN, Vpr, and possibly some structural proteins. Although CAp24 molecules dissociate during uncoating, some data suggest that they might play a role in infection of non-dividing cells. However, previous reports of NCp32 and MAp17 protein association with the PIC have been disputed. Recent data indicate that these proteins dissociate during uncoating. Cellular cyclophilin A (CypA) seems to play a critical role for proper core uncoating [62]. It is incorporated into virions through its interaction with CA. During entry, CypA protects the viral capsid from a human restriction factor (Ref1).

Reverse Transcription of HIV-1

Retroviruses are characterized by the process of reverse transcription, during which the single stranded vgRNA is converted into a dsDNA copy (Figure 5) [16, 19]. Initiation of reverse transcription occurs at the PBS. A host derived tRNA binds to the PBS and is used as a primer for reverse transcription [17, 114].

Reverse transcription is mediated by the virally encoded RT [16-18]. Evidence exists that reverse transcription might start prior to viral core insertion into the cytoplasm of the host cell, albeit it is widely accepted that the presence of deoxynucleotide 5'-triphosphates (dNTPs) in the cytoplasm is sufficient for initiation [58, 62, 63]. Regardless of when the process of reverse transcription initiates, the majority of the process occurs during RTC trafficking to the nucleus.

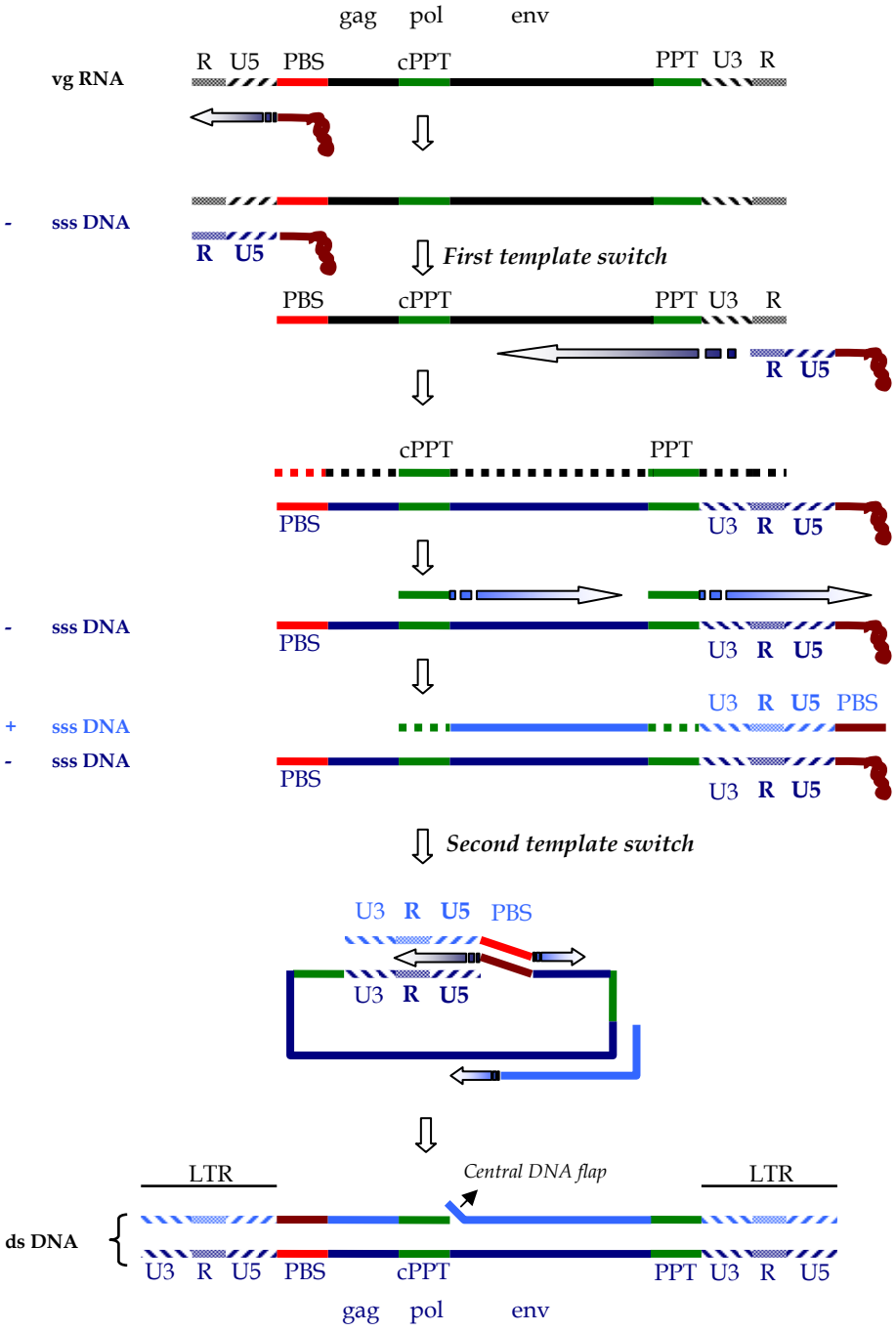

Figure 5. Model of HIV-1 reverse transcription.
Black lines represent viral genomic RNA (vgRNA), dark blue lines represent minus strand strong stop DNA (-sssDNA) while light blue lines represent plus strand strong stop DNA (+sssDNA). The primer binding site (PBS) in vgRNA (red). The central polypurine tract (cPPT), polypurine tract (PPT), U3, R, and U5 are indicated. The end result of reverse transcription is a dsDNA copy of the vgRNA. The central DNA flap is a result of initiation of +sssDNA synthesis from both the cPPT and PPT. Note that in the dsDNA product one PBS sequence is derived from the vgRNA (red) during –sssDNA synthesis, while the other is derived from the tRNA used to prime reverse transcription (dark red).

Minus Strand Strong Stop DNA (-sssDNA). In wild type HIV-1, -sssDNA synthesis is initialized from the 3' hydroxyl group of the host $tRNA^{Lys3}$ which is bound to the PBS [23, 58]. At this stage, RT acts as an RDDP using the vgRNA as the template for DNA synthesis. In this way, the R and U5 regions are generated. The $tRNA^{Lys3}$ primer remains bound to the newly synthesized DNA.

The First Jump or Translocation. Once past the tRNA bound to the PBS, the RNase H activity of RT will degrade the RNA used as template for polymerization of the -sssDNA [56, 58, 59]. The RNase activity of RT is crucial for the following step in reverse transcription. RNase H RT mutants cannot expose the -sssDNA, preventing what is referred to as the first jump or translocation [56]. The newly synthesized and exposed partial -sssDNA strand is then translocated from the 5' to the 3' end of the vgRNA via a strand transfer event. The jump is possible due to complementarity between the R regions of the vgRNA and –sssDNA [115]. Strand translocation most likely occurs both in *trans* and *cis*, meaning that the -sssDNA can translocate either to the RNA strand on which reverse transcription initiated, or to another vgRNA. In this way the virus can increase genetic variability [56].

Long, -sssDNA Synthesis. Minus strand strong stop DNA synthesis continues following the first jump. RT uses -sssDNA annealed to the 3' end of the vgRNA as a primer for elongation. During elongation, the RNA template of the newly formed RNA:DNA hybrid is digested by RNase H.

Plus Strand Strong Stop DNA (+sssDNA). Although RNase H digests most of the vgRNA used for negative strong stop synthesis, certain portions of the RNA are relatively resistant to hydrolysis [56]. This portion of the RNA, termed the polypurine tract (PPT), is located just upstream of the 3' U3. It is still unclear how the PPT remains resistant to cleavage. RT positioning seems to be responsible for protection, since the PPT sequence itself does not preclude it from digestion [116, 117]. Correct cleavage of the PPT has been attributed to its sequence, the unusual structure of the DNA at the PPT, and particular residues of RNaseH. The undigested RNA will then serve as primer for +sssDNA synthesis. RT synthesizes +sssDNA using –sssDNA as a template. Once RT initiates +sssDNA synthesis, the PPT is removed. Specifically, after the RT has synthesized 12 nucleotides, a second oppositely oriented RT cleaves the PPT at the PPT:U3 junction. The cleavage event releases the primer from the +sssDNA. As synthesis proceeds toward the 5' end of the –sssDNA, the RT copies the U3, R, U5, and the 3' 18 nucleotides of the tRNA primer used to initiate reverse transcription. If reverse transcription had been primed with $tRNA^{Lys,3}$, the methylated adenosine at position 58 (19^{th} nucleotide from the 3' end) is responsible for + sssDNA termination [118]. However, recent data using a complementation system in which *E.Coli* $tRNA^{Lys,3}$ (*Ec*$tRNA^{Lys3}$) is selected for reverse transcription, demonstrated that the methylated adenosine was expendable [119]. Nonetheless, it is important to note that the PBS is regenerated after the RT has copied the terminal 3' 18 nucleotides of the tRNA (used to initiate reverse transcription) that was still attached to the –sssDNA [18, 120].

Removal of the tRNA Primer. Following completion of the +sssDNA, the tRNA that was used to initiate reverse transcription is removed from the –sssDNA [56]. RNase H cleaves the tRNA at the DNA:RNA junction. However, a single adenosine often remains from the tRNA on the 5' end of the –sssDNA after the cleavage. This additional base does not seem to hinder downstream processes.

Second Translocation or Jump. Following removal of the tRNA primer from the –sssDNA, the 3' end of the +sssDNA is now free of interaction and available for binding to the 3' end of the –sssDNA [56]. The second translocation is made possible due to the complementarity of the PBS sequences of the –sssDNA (copied from vgRNA) and +sssDNA (copied from the tRNA used to prime reverse transcription). The result of the second jump is formation of a circular structure.

Completion of Reverse Transcription. After the second jump is complete, RT elongates both the –sssDNA and +sssDNA simultaneously [121]. At this stage, the RT acts as a DDDP, and finishes copying the rest of the proviral genome [58]. –sssDNA is used as a template to synthesize the missing portions of the +sssDNA and vice versa. The end result of reverse transcription is a linear dsDNA product. It is important to note the origin of the two PBSs in the viral dsDNA. The PBS of the –sssDNA is identical to the vgRNA PBS, while the +sssDNA PBS is derived from the tRNA primer used to initiate reverse transcription.

Multiple Plus Strand Initiation. In lentiviruses, in addition to the canonical PPT, an additional exact copy of the PPT is located in the IN portion of *pol* (termed central PPT or cPPT) [121-123]. This sequence was identified after it became apparent that there was a +sssDNA discontinuity near the middle of the HIV-1 dsDNA. The cPPT also remains undigested and bound to the -sssDNA and can be used to initiate +sssDNA synthesis with high efficiency in HIV-1. Additionally, mutation of the cPPT (while retaining the IN integrity) revealed that it was important in virus replication [124, 125]. Furthermore, an additional sequence called the central termination sequence (CTS) is located 90 nucleotides downstream of the 3' cPPT [126]. The CTS is crucial in causing synthesis termination during +sssDNA elongation. In case of multiple plus strand initiations, both the PPT and cPPT are necessary for successful reverse transcription. Moreover, two +sssDNA are synthesized. Following the second jump, RT elongates the +sssDNA from both +sssDNA fragments. Since the +sssDNA initiated from the PPT is upstream in relation to the +sssDNA initiated from the cPPT, it will eventually run into the alternatively primed +sssDNA. However, the RT synthesizing the PPT primed +sssDNA will not fall off. Instead, it will continue synthesizing past the cPPT of the –sssDNA and disengage once it reaches the CTS. During this process the RT will displace an approximately 89 to 99 nucleotide portion of the cPPT primed +sssDNA, creating an overlap dubbed the central DNA flap. Hence, the flap is located in the middle of the viral dsDNA. Since the dsDNA is eventually integrated into the host cell gene, the question is how this break in the provirus is resolved. Although it has only been shown *in vitro*, flap endonuclease 1 (FEN-1) removes the flap, while DNA ligase 1 repairs the break [127, 128]. The function of the central DNA flap has been controversial. It has been

implicated in both the proper completion of reverse transcription as well as in nuclear import of the preintegration complex (PIC) [56, 62, 129]. However, reexamination of the central DNA flap dismissed its necessity in PIC nuclear transport [130].

Nuclear Import of the Preintegration Complex (PIC)

A crucial step for successful infection by retroviruses is the incorporation of their reverse transcribed vgRNA into the host cell genome. In order to achieve this goal, viral dsDNA in the preintegration complex (PIC), must traffic through the cytoplasm and enter the nucleus.

Some retroviruses can only infect dividing cells, due to their inability to breach a fully intact nuclear membrane. On the other hand, lentiviruses are able to infect non-dividing cells, indicating that their PICs are somehow actively imported into the intact nucleus via a signal-mediated mechanism.

The eukaryotic nuclear membrane contains many NPCs that contact both the cytoplasm and the nucleoplasm, allowing bidirectional traffic. They are composed of 50 different nucleoporins, that are arranged to form and octagon [42]. The pathway for free diffusion is equivalent to a water filled channel with a diameter of 9 nm, albeit gold bead studies revealed that the pores can dilate up to a diameter of 25 nm. However, it is important to note that the HIV PIC, with a Stokes diameter of 56 nm, is much larger than the 25 nm central channel of the nuclear pore [131]. It is unknown how HIV overcomes this apparent size disparity.

Cytosolic nuclear pore receptor proteins (importins) mediate import through the NPC [42, 62]. They contain binding domains for both nucleoporins and proteins with a

nuclear localization sequence (NLS). Many NLSs consist of short stretches of positively charged amino acids lysine and arginine. NLS interaction with a nuclear pore receptor may occur directly, or via an adaptor protein. Importin 7 is an adapter protein that binds the receptor protein importin β and has been implicated in HIV PIC nuclear import [62].

Although the composition of the HIV PIC is still debated, INp32, MAp17, and Vpr have been implicated in nuclear import. Of the three, INp32 was considered the main mediator of nuclear import. Within its sequence it harbors a non-canonical NLS which is necessary and sufficient for nuclear translocation. However, new findings seem to have spurred a reevaluation of this function of INp32. Mutational analyses have shown that the NLS is not required for nuclear transport of INp32. Other contradictory studies have even suggested that INp32 does not interact with importins. The role of MAp17 and Vpr are also very controversial, since there are data both proving and disproving their involvement in PIC nuclear import. MAp17 contains both a canonical and non-canonical NLS sequence. Vpr does not contain an NLS, albeit it has been shown that it can directly bind proteins of the NPC. In addition to these three viral proteins, the central DNA flap has also been reported to play a role. The central DNA flap is a triple stranded DNA structure that possibly acts as a nuclear import signal either by adopting a conformation that allows it to bind components of the NPC, or to interact with cellular transport factors.

Integration

A key step in the HIV-1 life cycle is integration, a process in which the viral dsDNA is inserted into the host cell genome [56]. Following entry into the nucleus and

completion of reverse transcription, the dsDNA can have four fates: Formation of two LTR circles, formation of one LTR circles, autointegration and integration [95]. The first three scenarios represent unintegrated DNA forms. Two LTR circles are the result of blunt ligation of the 5' and 3' ends of the viral dsDNA and are indicative of HIV-1 entry into the nucleus. Formation of two LTR circles is mediated by the cellular non-homologous end-joining (NHEJ) mechanism whose physiologic function is to prevent apoptosis by repairing breaks in cellular dsDNA [132]. Homologous recombination between the 5' and 3' LTR produces a one LTR circle. Finally, viral dsDNA may autointegrate onto itself producing DNA with inversions and deletions. However, only integrated viral dsDNA will produce a productive infection.

Virally encoded IN mediates HIV-1 dsDNA insertion into the host cell genome [59]. IN is imported into the nucleus as a component of the PIC [62]. Integration can be subdivided into three distinct processes: 3' end processing, strand transfer and repair [23].

Once the preintegration complex has assembled, IN cleaves two terminal nucleotides from each 3' end of the blunt viral dsDNA. This event occurs at the highly conserved CA sequence, releasing a TT dinucleotide in most retroviruses. The cleavage results in the formation of recessed 3' and protruding 5' ends.

In the second step of integration, the exposed hydroxyl group at the terminal 3' CA attacks the phosphodiester bonds of host dsDNA via a Sn2-type reaction. In HIV-1, 5 nucleotides separate the attack sites directed from the two ends of the viral DNA. An esterification reaction joins the viral and host DNA. The newly formed phosphodiester bond between the viral dsDNA 3'CA and the host 5' DNA displaces the phosphodiester

bonds in the complimentary host DNA strand, creating short gaps between the cellular DNA and the unpaired bases at the 5' ends of the viral DNA.

Finally, the two unpaired bases at the 5' end of the viral DNA are removed and the gaps are filled in. Due to the mechanism of integration, the target site of integration is duplicated and exists at both ends of the provirus. These repair processes are presumed to be carried out by host cell repair mechanisms, although IN and RT involvement hasn't been ruled out. It seems that there is no specific site of integration.

Viral Gene Expression

The cellular transcription machinery transcribes viral genes using the integrated provirus as a template. General transcription factors (GTFs) bind to sequences in the 5' LTR and recruit RNA polymerase II (RNAPII) to the transcriptional start site [23, 35, 95]. Three Sp1 binding sites and the TATA box in the 5'U3 represent the major transcriptional promoter. NF-κB, AP1, and NFAT binding domains also aid in RNAPII positioning by recruiting their respective GTFs.

Following transcription in the nucleus, mRNAs undergo posttranscriptional modifications that include capping, 3' processing, polyadenylation and splicing. Transcribed vRNAs is no exception. However, virus viability depends on export of usvRNA. Therefore, HIV-1 has devised mechanisms to overcome this barrier. Even though they are transcribed from the same template, three types of vRNA are exported into the cytoplasm of the infected cell: usvRNA, ssvRNA, and msvRNA [21].

Early in viral gene expression, Nef, Tat and Rev are translated from msvRNA. Tat localizes to the nucleus where it recruits P-TEFb to the site of viral transcription [95].

It first binds the cyclyn T1 component of the complex and then the TAR of short viral RNA transcripts. This event allows the Cdk9 component of P-TEFb to hyperphosphorylate the CTD of RNAPII and increase processivity [96]. Overall, Tat increases the rate of vRNA transcription.

Rev also localizes to nucleus via its NLS, where it interacts with the RRE found in usvRNA and ssvRNA. Following binding and multimerization, Rev interacts with exportin 1 in the presence of RanGTP. Ran transports the complex to the NPC, where exportin 1 mediates export of Rev-bound usvRNA and ssvRNA through the nuclear pore.

The ssvRNA encodes gp160 and Vpu. Approximately 90% of the time, translation of usvRNA is terminated at the first stop codon, producing $Pr55^{Gag}$. In rare cases, a -1 frameshift occurs at the *gag-pol* junction resulting in the translation of the $Pr160^{Gag\text{-}pol}$.

Assembly, Release and Maturation

Virion production requires a coordination of events to occur in order to bring the necessary viral components together for assembly in the infected cell. The site of HIV-1 assembly is the cellular plasma membrane. Once all of the constituents are in place, the particle is then released from the cell through a budding process. The viral envelope is the cellular plasma membrane acquired by the budding virus. Following release, a maturation step takes place which renders the immature virion infectious.

$Pr55^{Gag}$ is largely responsible for assembly, budding and pinching off. The MA domain is responsible for $Pr55^{Gag}$ targeting to the cellular plasma membrane [23]. Although sequences within MA contribute to membrane binding, the major culprit seems

to be a postranslationally attached myristic acid to the N-terminal Gly. Exposure of this modification seems to be dependent on multimerization of $Pr55^{Gag}$.

$Pr55^{Gag}$:$Pr55^{Gag}$ and $Pr55^{Gag}$: $Pr55^{Gag\text{-}pol}$ interactions are mediated by the CA CTD. *In vitro* expression of purified CAp24 results in assembly of tubular particles [61, 66]. However, NC has also been implicated in $Pr55^{Gag}$ multimerization, since CAp24-NCp7 fusion proteins necessitate a lower concentration for self assembly [133]. Moreover, assembly was shown to be more efficient in the presence of RNA, indicating that RNA might be involved in initiation or organization of the process.

HIV-1 genomes are selected from the cellular milieu that contains a myriad of cellular RNA, usvRNA, ssvRNA and msvRNA [60]. Essentially, encapsidated usvRNA is vgRNA. The selection is mediated by the $Pr55^{Gag}$ NC binding to usvRNA Ψ. NC contains two zinc finger domains that bind RNA [71]. The Ψ site has been previously mapped to approximately 120 nucleotides upstream of the *gag-pol* frameshift site. However, recent data suggest that the R, U5, PBS and the *gag* coding regions also contain sequences involved in packaging [60]. The *gag* leader region is believed to form a series of four stem loop structures (SL1, SL2, SL3, SL4) that might have overlapping functions. SL1 contains the primary DIS, which is imperative for efficient genome packaging [34, 60]. This site forms a kissing loop through its GC-rich stem loop [60]. Although NC weakly binds to SL1, it is thought to promote formation of a more stable duplex species in comparison to the kissing dimer by destabilizing the SL1 stem loop. NC binds SL2 and SL3 with high affinity. Moreover, SL2 contains the major splice donor site. Upon splicing, the splice donor site is destroyed, indicating that this might be

involved in discriminating between spliced (ssvRNA and msvRNA) and usvRNA. SL4 is only weakly bound and its function is unresolved.

Although the mechanism of NC mediated packaging of usvRNA is well established, the fate of usvRNA following export from the nucleus is a point of contention. Conceivably the there are three possible scenarios: 1. There are two separate pools of usvRNA one of which is drawn from for encapsidation, while the other is used for translation 2. The usvRNA is used interchangeably for either translation or encapsidation, albeit translation is not required for encapsidation 3. Encapsidation of usvRNA necessitates prior translation. So far, determination of the usvRNA fate involved biochemical assays in which infected cells would be treated with transcription or translation inhibitors of vRNA. Based on these methods, it was concluded that MuLV follows scenario 1, HIV-1 follows scenario 2, while HIV-2 follows scenario 3 [134-136]. Although Butsch and Boris-Lawrie provide compelling evidence that translation is not required for encapsidation of HIV-1 usvRNA, the downfall of the system is that translation cannot be completely abrogated in cell-based systems [136]. Therefore, coordination of translation and vgRNA encapsidation can still be a possibility. Moreover, recent data provide evidence of *cis* packaging of usvRNA by nascent $Pr55^{Gag}$ in HIV-1[137]. Additionally, HIV-2 packages translated usvRNA [134, 135]. Although possible, it would be very surprising if two closely related retroviruses (HIV-1 and HIV-2) developed different mechanisms for such a crucial step in their life cycle.

In order for the virion to be infectious, gp120 /gp41 trimers need to be present at the assembly site. Virus attachment to the target cell and the subsequent fusion are mediated by gp120 and gp41, respectively [23]. Following translation of ssvRNA at the

rER, gp160 is transported through the Golgi where it is cleaved by a cellular furin protease forming gp120 and gp41. These two glycoproteins associate via weak noncovalent bonds after cleavage. During transport to the plasma membrane, the glycoproteins are heavily glycosylated. Although the mechanism of gp120/gp41 incorporation into the budding virions is undefined, evidence suggests that it is mediated through a direct interaction of gp41 with MA.

Budding and release are the final step in producing immature virions. They are mediated by the $Pr55^{Gag}$ p6 domain. A highly conserved Pro-Thr-Ala-Pro (PTAP) motif is responsible for the release function. Autoproteolysis of PR from $Pr160^{Gag\text{-}pol}$ precursor produces free aspartic PR that cleaves $Pr55^{Gag}$ at two, and $Pr160^{Gag\text{-}pol}$ at five sites[58]. Further cleavage of NCp15 and RTp66 marks the end of maturation which renders HIV-1 infectious [59].

HIV-1 tRNA Primer Selection

Retroviruses and Their Preferential tRNA Primers

The mechanism of tRNA primer selection is still not completely understood. As is the case with other retroviruses, HIV-1 uses a host tRNA to initiate reverse transcription [17, 114]. There is no common tRNA isoacceptor used by all retroviruses (Table 1). For example, $tRNA^{Trp}$ is used by members of the avian sarcoma and leucosis virus group; $tRNA^{Pro}$ is used by murine leukemia virus (MuLV); HIV-1 and HIV-2, as well as SIV use $tRNA^{Lys3}$ [14, 37, 39, 83, 138]. There does not seem to be a correlation between similarity of retroviral genomes and their preferential tRNA isoacceptor used for reverse transcription[59]. Annealing of the 3' terminal 18 nucleotides of the cognate

tRNA to the PBS is necessary for the initiation of reverse transcription. Although most cellular tRNAs have been found in the HIV-1 virion, retroviruses exhibit preferential packaging of their cognate tRNA primer [58]. HIV-1 preferentially incorporates $tRNA^{Lys}$ [139]. There are approximately 8 molecules of $tRNA^{Lys3}$ and 12 molecules of $tRNA^{Lys1,2}$ per pseudodiploid genome, albeit only $tRNA^{Lys3}$ binds tightly to the genome [139-141].

Significance of the PBS

The PBS is crucial for initiation of reverse transcription. Deletion of the PBS results in virus that is unable to bind $tRNA^{Lys3}$ and initiate reverse transcription [142]. Further deletional analyses demonstrated that the first five nucleotides of the PBS are absolutely necessary for primer binding. Moreover, deletions in the middle and 3' end of the PBS result in a infectious virus that had severely impaired replication kinetics [142, 143]. Mutating the PBS to be complementary to an alternative tRNA species resulted in viruses that were able utilize the alternative tRNA for replication, although they eventually reverted to using $tRNA^{Lys3}$ as a primer for reverse transcription [144-148].

Significance of the A-loop

Multiple studies provide evidence of interaction between the tRNA primer and the vgRNA outside the PBS [58]. Two adenosine-rich sequences were identified upstream and downstream of the PBS that could putatively interact with the anticodon of the tRNA primer. Through mutational, nuclease and chemical footprinting analyses, it was determined that only the adenosine-rich sequence (A-loop) 5' to the PBS interacts with the anticodon of $tRNA^{Lys3}$ [57]. Moreover, it was found to be conserved among different

HIV-1 isolates. Deletion of the A-loop significantly influences infectivity and reverse transcription efficiency. Interestingly, this sequence is partially regenerated after culturing. Viruses with a partially regenerated A-loop exhibit replication kinetics similar to those of wild type HIV-1 [149]. Through mutational studies, our lab has established the importance of the PBS and the A-loop in primer selection [142-145, 150-156]. HIV-1 clones with PBS and A-loop complementarity to $tRNA^{Lys1,2}$, $tRNA^{His}$, $tRNA^{Met}$, $tRNA^{Glu}$, and $tRNA^{Thr}$ stably use their respective tRNA as primers in reverse transcription. Alternatively, $tRNA^{Ile}$, $tRNA^{Ser}$, and $tRNA^{Tyr}$ could not be continuously selected even with appropriate A-loop modifications. Therefore, compensatory mutations of the A-loop to make them complementary to the anticodon of the non-cognate tRNA primer, result in a more stable usage of the specific tRNA isoacceptor only in certain mutant viruses [143, 144, 157-159]. In an attempt to resolve this discrepancy additional mutational studies were employed and will be discussed later.

Significance of tRNA Biogenesis

The mere fact that tRNAs are used as primers for retroviral reverse transcription, suggested the involvement of the cellular protein biosynthetic pathway in primer selection. Since it is difficult to manipulate endogenous levels of tRNA in mammalian cells, previous studies have utilized a HIV-1 mutant that requires a non-mammalian tRNA for infectivity. $HXB2^{yPhe}$ utilizes yeast $tRNA^{Phe}$ ($ytRNA^{Phe}$) to initiate reverse transcription if this tRNA is provided *in trans* [153, 160]. Later studies involving $HXB2^{yPhe}$ demonstrated that transfected $ytRNA^{Phe}$ is aminoacylated and enters the cell's protein biosynthesis machinery [161]. Further investigation of this system revealed that

ytRNA^{Phe} lacking the D-loop (hence defective for nuclear export) was retained in the nucleus and did not rescue the HXB2^{yPhe} virus. However, transfection of this defective tRNA directly into the cytoplasm restored infectivity of HXB2^{yPhe}. Additionally, a mutant ytRNA^{Phe} that was unable to be aminoacylated (ytRNA^{Phe}UUA), was transported to the cytoplasm and was able to complement infectivity of HXB2^{yPhe}, although at a level lower than was observed with wild type ytRNA^{Phe}. Taken all together, these data support the idea that tRNA capture occurs in the cytoplasm after nuclear export as well as that primer selection is linked to protein synthesis at the level of translation.

HIV-1 Models of Primer Selection

Model 1. Based on data from the field, we have developed a working model of HIV-1 primer selection (Figure 6). During translation of usvRNA, a large RNA stem loop structure at the *gag-pol* junction causes ribosomes to stall. Ribosomal pausing may increase the local accumulation of translational machinery elements and therefore reduce the rate of protein synthesis. Increased local concentration of aminoacylated tRNA^{Lys3} bound to eEF1A and its subsequent deacylation following exit from the ribosome, could provide the opportunity for HIV-1 to capture the tRNA before it is reacylated by the appropriate synthetase.

This model is supported by recent data. Codons corresponding to tRNAs continuously used for priming reverse transcription were all found upstream of the HIV-1 frameshift site [162]. Moreover, out of five lysine codons found in the region, three

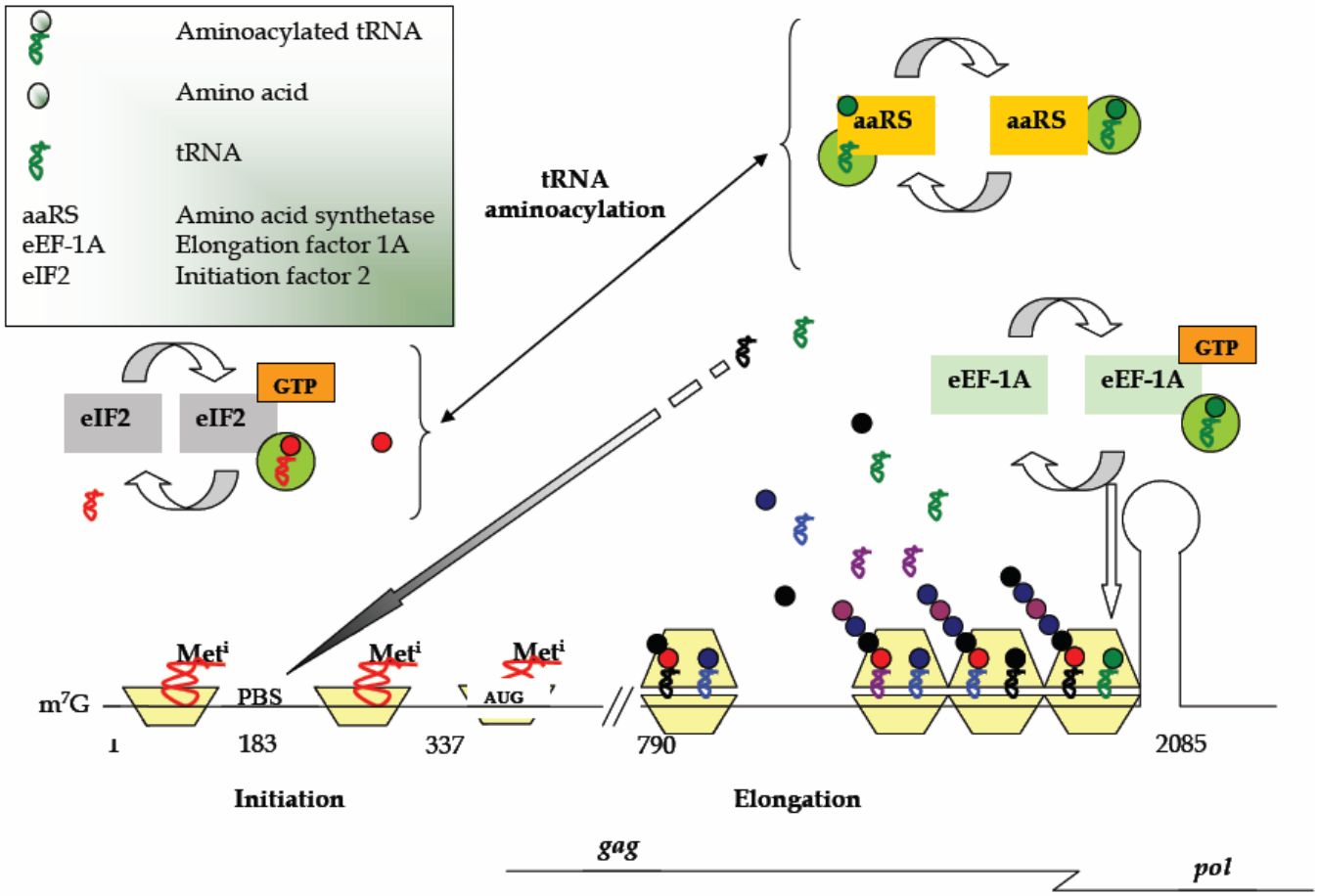

Figure 6. Schematic representation of primer selection Model 1 (Frameshift model).
Translation of viral genomic RNA (vgRNA) is shown. Both initiation and elongation phases of protein biosynthesis are represented, along with the components of the initiation ternary complex (eIF2-GTP-tRNAMet(i)) and the elongation ternary complex (eEF-1A-GTP-tRNA^{AA}). During translation of vgRNA, the ribosomes stall at the *gag-pol* junction due to a large RNA stem loop, and in 10% of cases a -1 frameshift occurs causing synthesis of Pr160$^{Gag\text{-}pol}$ instead of Pr55^{Gag}. During this ribosomal pausing, a local increase of the biosynthetic machinery might allow a local increase of tRNAs free from any interaction (usually complexed with protein, amino acid, or both) to be selected and captured by the virus (indicated by large black block arrow).

correspond to $tRNA^{Lys3}$, while the other two correspond to $tRNA^{Lys1,2}$. An HIV-1 clone was constructed where all of the $tRNA^{Lys3}$ codons were mutated to correspond to $tRNA^{Lys1,2}$. This clone was dubbed the F5 frameshift mutant. Whereas the NL4 clone with just A-loop and PBS (NL-4Lys1,2-AC) complementarity to $tRNA^{Lys1,2}$ exhibited poor replication kinetics, engineering a PBS and A-loop complementary to $tRNA^{Lys1,2}$ into the frameshift mutant (NL-4Lys1,2-AC-F5) rescued replication to levels approaching those of wild type virus (NL4-WT). These results support the idea that a local microenvironment enriching for particular tRNA was important in primer selection. Moreover, it linked primer selection with translation of vgRNA. Nonetheless, other factors might also play a role in this process of primer capture.

Model 2. $tRNALys^{1,2,3}$ are all aminoacylated by lysyl tRNA synthetase (LysRS) in the cellular biosynthetic pathway. Both the $tRNA^{Lys}$ isoacceptors and LysRS are selectively packaged into HIV-1 virions [139, 163]. Expression of processed or unprocessed $Pr55^{Gag}$ results in the formation of virus-like particles (VLPs) which are enriched for LysRS. However, only coexpression of $Pr55^{Gag}$ with $Pr160^{Gag\text{-}pol}$ produces VLPs that selectively package both LysRS and $tRNA^{Lys3}$ [164]. The conclusion from these studies was that $Pr55^{Gag}$ or portions of it, bind LysRS, while the Pol region of $Pr160^{Gag\text{-}pol}$ is responsible for binding $tRNA^{Lys3}$. Previous studies had shown that the RT portion of $Pr160^{Gag\text{-}pol}$ specifically bound $tRNA^{Lys3}$; while HIV-1 mutants lacking RT showed a log decrease in $tRNA^{Lys3}$ packaging [141, 165]. Both NCp7 and $Pr160^{Gag\text{-}pol}$ NC have the ability to bind RNA through their zinc finger motifs [59]. Moreover, NC

has a high binding affinity for a TΨC loop of $tRNA^{Lys3}$ as well as to the noncoding region 3' of the PBS [59].

Based on this data, Kleiman's group has proposed an HIV-1 primer selection model that involves binding of $Pr55^{Gag}$ to LysRS and the RT portion of $Pr160^{Gag\text{-}pol}$ binding to $tRNA^{Lys3}$ [166]. The $Pr55^{Gag}$-LysRS complex also interacts with $Pr160^{Gag\text{-}pol}$ which binds the $tRNA^{Lys3}$. Simultaneous binding of the $Pr160^{Gag\text{-}pol}$ to $tRNA^{Lys3}$ and $Pr55^{Gag}$ to $tRNA^{Lys3}$ serves not only to stabilize the complex, but also signal its packaging into the virion. Following transport of the primer complex into the budding virion, the tRNA primer is positioned onto the PBS, presumably following maturation.

However, there are multiple lines of evidence that are not supported by this model of primer selection. The group itself identified selective packaging of $tRNA^{Ile}$ and $tRNA^{Asn}$ which seem to serve no purpose [141]. Although $tRNA^{His}$ is not selectively packaged, a HIV-1 mutant with PBS complementarity to $tRNA^{His}$ replicates in culture [154]. Moreover, HIV-1 also selectively packages $tRNA^{Lys1,2}$, albeit HIV mutants with the PBS complementary to $tRNA^{Lys1,2}$ revert to using $tRNA^{Lys3}$ in culture [145, 146, 148]. Although compensatory A-loop mutations of this virus allow continual selection of $tRNA^{Lys1,2}$, replication is severely compromised [159]. Finally, previous reports indicating the specificity of $Pr160^{Gag\text{-}pol}$ RT binding to $tRNA^{Lys3}$ has been disputed [167, 168].

Mechanics of Translation

Translation is a highly organized and complex process that converts mRNA into protein. It can be broken down into four distinct phases: initiation, elongation,

termination, and recycling [169]. A current model of the first three phases is schematically represented in Figure 7. The first step in the initiation phase is the formation of the initiation ternary complex. This complex consists of eIF2 bound to GTP and initiator methionine (eIF2-GTP-$tRNA^{Met(i)}$). eIF2 affinity for GDP is approximately 100 fold greater than for GTP. These thermodynamically unfavorable conditions are overcome by the action of eIF2B. Once the initiation ternary complex has formed, it binds the small ribosomal subunit (40S). Some of the proteins involved in this process are eIF 1, 1A and 3. The ternary complex bound to the mRNA is referred to as the 43S complex. Next, the eIF4F complex binds to the 7 methylguanosine cap structure of the mRNA. eIF4F consists of eIF 4A, 4E and 4G. eIF4A is a DEAD-box RNA dependent ATPase that can unwind RNA duplexes in vitro. eIF4E binds to the cap structure of mRNA, while eIF4G interacts with the poly (A) binding protein (PAB). Eukaryotic mRNA have a poly(A) tail located at their 3' ends. It is proposed that the interaction between eIF4G and PAB circularizes mRNA as a quality control mechanism.

Other factors include eIFs 4B and 4H, which are RNA binding proteins. Through the unwinding action of eIF4A, the 43S complex moves down the RNA in search of the start codon located in a favorable sequence context (Kozak sequence). Base pairing occurs between the anticodon of $tRNA^{Met(i)}$ and the codon sequence of the mRNA. This, in turn, activates GTP hydrolysis by eIF2 aided by GTPase-activating protein (GAP) eIF5. At some point, eIF5B-GTP binds to the complex and facilitates binding of the large ribosomal subunit (60S). Hydrolysis of the high energy bond releases eIF5B-GDP from the complex. This event presumably ends initiation and signals the elongation phase of protein biosynthesis [169].

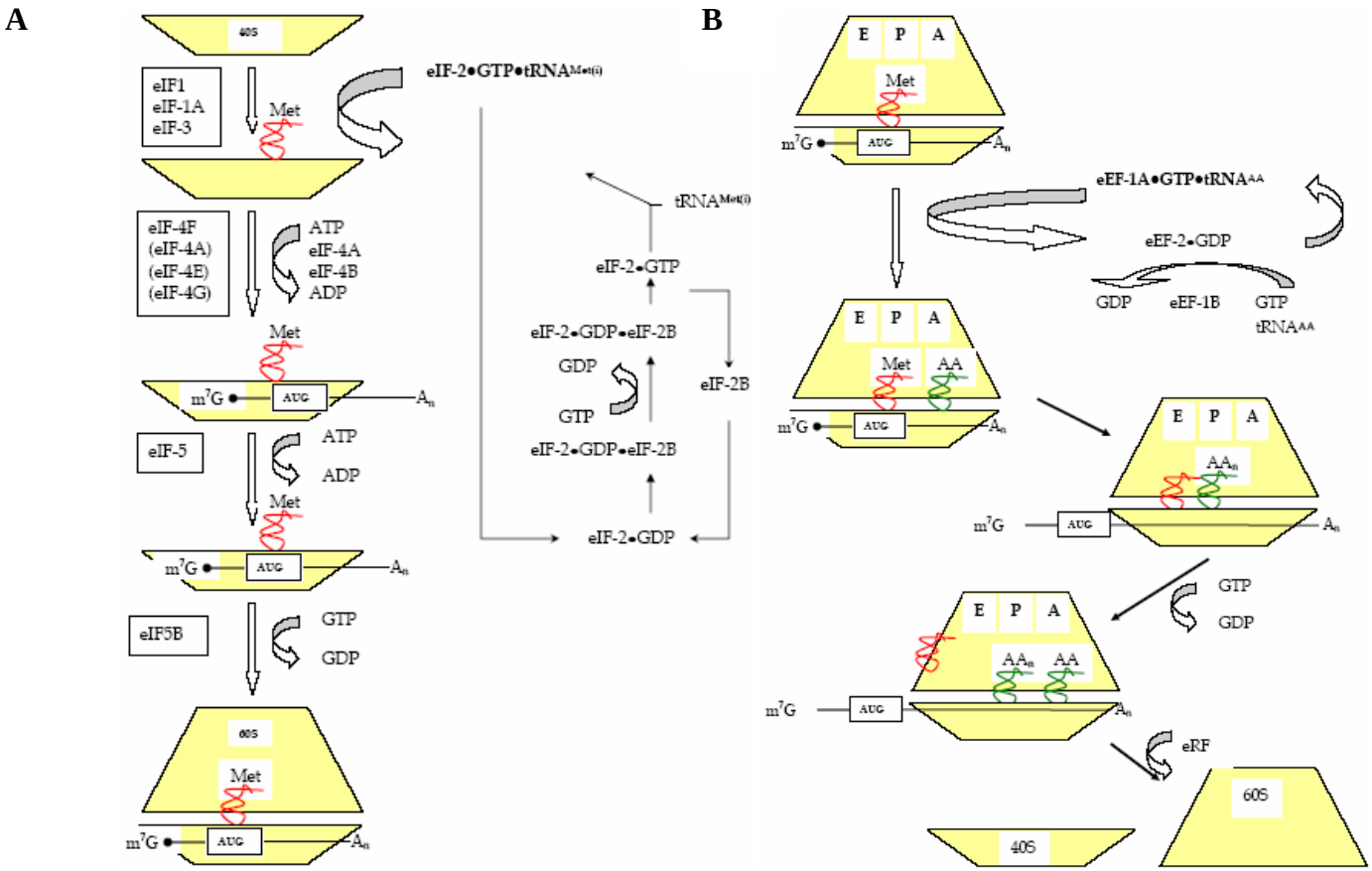

Figure 7. Schematic representation of cellular translation.

Panel A. Translational initiation. The inset shows formation of the initiation ternary complex. This complex delivers initiator methionine tRNA ($tRNA^{Met(i)}$) to the small ribosomal subunit. This structure binds mRNA, scans it, and begins translation once it encounters an AUG codon in a favorable context (Kozak sequence).

Panel B. Translation elongation and termination. Formation of the elongation ternary complex (eEF-1A-GTP-$tRNA^{AA}$) is shown. During elongation, tRNAs enter the ribosomal A site and a peptide bond forms between the amino acid attached to the tRNA in the A site and the amino acid attached to the tRNA already located in the P-site. The ribosome shifts and the tRNA from the P-site enters the E site, form which it is ejected on the following round. This process repeats (allowing the polypeptide chain to grow) until a stop codon is exposed in the A site. A release factor (eRF1) decodes stop codons (UAA, UAG, or UGA). It enters the A site, causing hydrolysis the ester bond that links the polypeptide chain to the tRNA as well as ribosome disassembly.

In the elongation phase, amino acids will be added to the growing polypeptide chain according to the codon sequence of the mRNA. Once a codon is available in the aa-tRNA binding site (A site) on the 40 S subunit of the ribosome, an elongation ternary complex consisting of aminoacylated tRNA and GTP bound to eEF1A (aa-tRNAeEF1A-GTP) will enter the ribosome. In a testing phase, the anticodon of the tRNA will try to make an interaction with the mRNA codon. If successful, eEF1A-GTP is hydrolyzed and eEF1A-GDP leaves the ribosome. The exchange of GDP to GTP in the eEF1A is facilitated by eEF1B. The tRNA 3' CCA enters the A site of the large ribosomal subunit, where a peptide bond is formed between the incoming amino acid and the peptide present in the peptidyl-tRNA binding site (P site). tRNAs present in the ribosome are in mixed hybrid states. Newly formed peptidyl tRNA is in the A/P state, and the deacylated tRNA is in the P/E state (exit site-E site). At this point eEF2-GTP enters a site similar to the one used for the "testing phase" by the ternary complex. This event physically forces the tRNAs completely into the P and E sites respectively as well as moving the mRNA by 3 nucleotides. GTP is hydrolyzed in the GTPase center of the ribosome, and eEF2-GDP leaves the ribosome. These steps occur continuously, until a stop codon is exposed in the A site [170]. This event marks the end of elongation and the start of termination.

A release factor, eRF1, decodes three possible stop codons – UAA, UAG or UGA. eRF1 binding has the net result of hydrolysis of the ester bond linking the polypeptide chain to the tRNA located in the P site of the ribosome, which is believed to be catalyzed by the peptidyl transferase center of the ribosome. The ribosome disassembles and the polypeptide is released [169].

tRNA and Aminoacylation

In eukaryotic organisms, tRNA is transcribed by the cellular DDRP III in the nucleus [42, 171]. The primary transcript (precursor) needs to be modified in order to produce a mature tRNA. Some of the modifications include cleavage of the 5' and 3' extensions termed the leader and trailer, respectively, addition of CCA to the 3' end of the tRNA, intron splicing in some tRNA, and numerous nucleotide modifications [171-173]. In the first tRNA processing step, RNase P cleaves the 5' leader sequence in a yet unknown mechanism. Following this event, endonucleases RNase E and RNase III cleave the middle of the 3' trailer. Exonucleases RNase II, RNase BN', and RNase PH degrade the residual 3' sequence. Recently, another endonuclease, RNase Z, was found to cleave the 5' side of the CCA sequence. Following this cleavage, the CCA-adding enzyme regenerates and/or repairs the CCA in a template independent manner using CTP and ATP as substrates [171]. All known tRNAs contain the 3' terminal sequence. This sequence is a necessary requirement for the addition of amino acid to the 3' end of the tRNA, as well as interaction with the large ribosomal subunit during translation. At this point, tRNAs adopt the canonical L-shape form, and are often schematically depicted in literature as a cloverleaf. Functional domains that can be identified are the acceptor stem, the D-arm, the anticodon and the TΨC loop. The acceptor stem is the location where the cognate aminoacyl tRNA synthetase adds the correct amino acid to the tRNA. The anticodon pairs with the codon of the mRNA in the A site of the small ribosomal subunit during the process of translation. However, additional modifications are necessary for complete maturation of tRNA. Specific nucleotides on the tRNA are modified. Mapping the modifications on the 50S ribosomal RNA shows that all of them are located around

the peptydyl-transfer center. Functionally, these modifications are divided into two groups. The first are modifications of the tRNA core region formed by the D and TΨC loop, and the second are located in the anticodon loop. The former modifications are implicated in the L-shaped tRNA tertiary stability, while the latter have dual functions: correct anticodon-codon interaction and recognition of the cognate aminoacyl tRNA synthetase [171]. Recently it has been shown that tRNA aminoacylation by tRNA synthetase occurs in the nucleus [174]. It has been postulated that nuclear aminoacylation provides a proofreading mechanism, so that defective tRNA are not exported from the nucleus. Molecular mechanisms of tRNA aminoacylation have been deciphered for isoleucyl tRNA [175, 176]. Charging of the tRNA occurs in a two-step process. Isoleucyl tRNA synthetase has two catalytic sites – a synthesis and an editing site. The synthesis site prevents larger amino acids to bind to the site. Additionally, the binding affinity of the correct amino acid to its synthesis site is highest in comparison to other amino acids. However, a second proofreading mechanism is activated after the amino acid has been covalently bound to AMP. tRNA binding to the synthetase results in two scenarios. If the correct amino acid is in the synthesis site, tRNA is charged. However, if the incorrect amino is bound to the synthetase it is translocated to the editing site where it hydrolyzed from AMP and released [42, 173, 177, 178]. Additionally, it has been shown that aminoacylation aids in tRNA export out of the nucleus, but is not absolutely necessary [174, 179]. Exportin-t is a member of the vertebrate importin-β family of proteins. It specifically binds tRNA in a Ran (GTPase) dependent manner, as well as nuclear pore proteins, facilitating the export of tRNA from the nucleus [172, 180]. Exportin-t binding to tRNA is another level of control, since abnormally processed tRNA

are not exported efficiently [172, 174, 179]. However, there is at least another mechanism of tRNA export into the cytoplasm, since inhibition of exportin-t pathway doesn't completely abolish tRNA transport [179]. Exportin 5 is another member of the importin-β family of proteins that binds aminoacylated and non-aminoacylated tRNA in Ran-GTP dependent manner and facilitates export [172].

Specialized tRNA: $tRNA^{Met(i)}$

Eukaryotes harbor two different species of methionine tRNAs – initiator ($tRNA^{Met(i)}$) and elongator ($tRNA^{Met(e)}$). As their respective names indicate, $tRNA^{Met(i)}$ is used exclusively for initiation, while $tRNA^{Met(e)}$ is used in decoding methionine codons during the elongation phase of protein synthesis [181, 182]. The two different tRNAs bind a completely different set of proteins during translation. While $tRNA^{Met(i)}$ binds initiation factors (eIF2) and is responsible for identifying the translational start of the protein, $tRNA^{Met(e)}$ binds elongation factors (eEF1A). The two methionine tRNAs have the same anticodon sequence 5' CAU 3', and are charged with the same aminoacyl synthetase. However, nucleotide differences dictate differences in structure and function [182, 183]. Namely, a A1:U72 base pair at the end of the acceptor stem, three consecutive G:C base pairs in the anticodon stem (G29G30G31:C39C40C41) and A54 and A60 instead of T54 in the TΨC loop of $tRNA^{Met(i)}$ all contribute to the functional alikeness.

Significance of Dissertation Research

HIV is the causative agent of AIDS. Since its identification in 1983, an estimated 65 million people have been infected with HIV, out of which 25 million people have died as a consequence of AIDS [15]. The AIDS pandemic has proven to be the defining medical and public health issue our time [8]. Therefore, a concerted world effort has been put forth in studying this relatively new disease causing agent. Extraordinary progress has been made in the last 25 years, resulting in defining many aspects of the viral life cycle. In turn this research led to identification of potential drug targets. There are over 20 FDA approved antiretroviral drugs on the market today which have transformed a lethal infection into a chronic, relatively treatable disease [184]. However, most of these drugs target only HIV reverse transcriptase and protease. Due to the high mutation rate of HIV, there is a fear of resistant strain emergence [24, 25]. Consequently, it is important to explore new treatment possibilities. The primer binding site (PBS) is one of the most conserved sequences in the viral genome and presents a viable target. Elucidating intricacies of the HIV-1 primer selection mechanism, could lead to designing compounds that would prevent tRNA primer placement on the PBS thereby abrogating new rounds of infection.

The two reports presented in this dissertation provide evidence of coordination between primer selection, translation of usvRNA and encapsidation. The conclusions drawn in these reports were the result of mutational studies of the R-U5-PBS region of HIV-1. In the first paper we described three PBS mutants with complementarity to elongator methionine tRNA ($tRNA^{Met(e)}$), initiator methionine tRNA ($tRNA^{Met(i)}$) and initiator methionine with an A to G point mutation at position 13 $tRNA^{Met(i)AG}$. The

viruses were named HXB2-Met(e), HXB2-Met(i) and HXB2-Met(i)AG. Confirming previous findings, HXB2-Met reverted to using $tRNA^{Lys3}$ following extended culturing. Unexpectedly, HXB2-Met(i) could not produce a productive infection in human Caucasian T cell lymphoma (SupT1) cells. However, relative amounts of $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$ were equivalent in both the producer and target cells, indicating that this was not the cause of the defect. Moreover, an endogenous reverse transcription assay demonstrated that $tRNA^{Met(i)}$ could be selected and used for reverse transcription. Therefore, we sought to characterize HXB2-Met(i). This virus was poorly infectious. The surprising result was that transfection of the HXB2-Met(i) proviral clone produced lower overall amounts of virus, a phenotype that was never previously observed with any HIV-1 PBS mutants. A western blot of the virions confirmed our previous finding, but also that cleavage of $Pr55^{Gag}$ was not the cause of the defect. Discovery of the artificially introduced Kozak AUG in the HXB2-Met(i) PBS, led to the construction of HXB2-Met(i)AG in which this AUG was mutated to a GUG. This point mutation rescued not only virus production but also infectivity. Moreover, HXB2Met(i)AG was able to productively infect SupT1 cells. The most interesting result was procured from sequencing the PBS of integrated HXB2-Met(i)AG. It confirmed that $tRNA^{Met(i)}$ was used in reverse transcription. Most importantly, it demonstrated a preferential encapsidation of translated usvRNA. Together these results linked primer selection, viral translation and vgRNA selection. The second paper defined the relative contribution of the PBS, A-loop and the codon usage upstream of the frameshift region in primer selection. Our experimental approach consisted of testing three groups of HIV-1 virions.

The groups were divided based on PBS complementarity to $tRNA^{Lys1,2}$, $tRNA^{Lys3}$, and $tRNA^{Met}$ (Table 2). Within each group we made additional A-loop and/or frameshift

Table 2. Tested HIV-1 viral clones. Names are derived based on PBS, A-loop and frameshift region complementarity

Virus	PBS	A-loop	Frameshift
NL-4Lys1,2	$tRNA^{Lys1,2}$	$tRNA^{Lys3}$	WT
NL-4Lys1,2-AC	$tRNA^{Lys1,2}$	$tRNA^{Lys1,2}$	WT
NL-4Lys1,2-F5	$tRNA^{Lys1,2}$	$tRNA^{Lys3}$	F5
NL-4Lys1,2-AC-F5	$tRNA^{Lys1,2}$	$tRNA^{Lys1,2}$	F5
NL-4WT	$tRNA^{Lys3}$	$tRNA^{Lys3}$	WT
NL-4WT-Lys1,2-AC	$tRNA^{Lys3}$	$tRNA^{Lys1,2}$	WT
NL-4WT-F5	$tRNA^{Lys3}$	$tRNA^{Lys3}$	F5
NL4WT-Lys1,2-AC-F5	$tRNA^{Lys3}$	$tRNA^{Lys1,2}$	F5
NL-4Met-Lys1,2-AC	$tRNA^{Met}$	$tRNA^{Lys1,2}$	WT
NL-4Met-Lys1,2-AC-F5	$tRNA^{Met}$	$tRNA^{Lys1,2}$	F5

region mutations. In the first group, NL-4Lys1,2 and NL-4Lys1,2-F5 replicated poorly and reverted to using $tRNA^{Lys3}$ as a primer for reverse transcription. Conversely, reversion was not observed in NL-4Lys1,2-AC and NL-4Lys1,2-AC-F5 infected cultures. However, the former virus exhibited poor replication kinetics, while the latter replicated almost as well as NL-4WT. In the second group, viruses had PBS complementarity to $tRNA^{Lys3}$ (wild type). We were unable to force the conversion of the PBS to be complementary to $tRNA^{Lys1,2}$, even in NL-4WT-Lys1,2-AC-F5. In this virus, both the A-loop and the frameshift region are mutated so that they would interact with $tRNA^{Lys1,2}$. In the third group, we attempted to force viruses with PBS complementarity to $tRNA^{Met}$ to use $tRNA^{Lys1,2}$. Previous studies have shown that NL-4Met reverts to using $tRNA^{Lys3}$ as a primer, although a virus intermediate with PBS complementarity to $tRNA^{Lys1,2}$ has been

isolated. Both NL-4Met-Lys1,2-AC and NL-4Met-Lys1,2-AC-F5 replicated poorly and could not be forced to use $tRNA^{Lys1,2}$ as a primer for reverse transcription. Taken together, the results from this study demonstrate that the codon usage upstream of the *gag-pol* frameshift site influence replication, with the caveat that the PBS is the major determinant of primer usage, while the A-loop is responsible for continuous selection of a tRNA primer.

ANALYSIS OF THE REPLICATION OF HIV-1 FORCED TO USE $tRNA^{Met(i)}$ SUPPORTS A LINK BETWEEN PRIMER SELECTION, TRANSLATION AND ENCAPSIDATION

by

UROS V. DJEKIC AND CASEY D. MORROW

Retrovirology 4:10

Abstract

Background

Previous studies have suggested that the process of HIV-1 tRNA primer selection and encapsidation of genomic RNA might be coupled with viral translation. In order to further investigate this relationship, proviruses were constructed in which the primer-binding site (PBS) was altered to be complementary to elongator $tRNA^{Met}$ ($tRNA^{Met(e)}$) (HXB2-Met(e)) or initiator $tRNA^{Met}$ ($tRNA^{Met(i)}$) (HXB2-Met(i)). These $tRNA^{Met}$ not only differ with respect to the 3' terminal 18-nucleotides, but also with respect to interaction with host cell proteins during protein synthesis.

Results

Consistent with previous studies, HXB2-Met(e) were infectious and maintained this PBS following short-term *in vitro* culture in SupT1 cells. In contrast, transfection of HBX2-Met(i) produced reduced amounts of virus (as determined by p24) and did not establish a productive infection in SupT1 cells. The low infectivity of the virus with the PBS complementary to $tRNA^{Met(i)}$ was not due to differences in endogenous levels of cellular $tRNA^{Met(i)}$ compared to $tRNA^{Met(e)}$; $tRNA^{Met(i)}$ was also capable of being selected as the primer for reverse transcription as determined by the endogenous reverse transcription reaction. The PBS of HXB2-Met(i) contains an ATG, which could act as an upstream AUG and syphon scanning ribosomes thereby reducing initiation of translation at the authentic AUG of Gag. To investigate this possibility, a provirus with an A to G change was constructed (HXB2-Met(i)AG). Transfection of HXB2-Met(i)AG resulted in increased production of virus, similar to that for the wild type virus. In contrast to

HXB2-Met(i), HXB2-Met(i)AG was able to establish a productive infection in SupT1 cells. Analysis of the PBS following replication revealed the virus favored the genome with the repaired PBS (A to G) even though $tRNA^{Met(i)}$ was continuously selected as the primer for reverse transcription.

Conclusion

The results of these studies suggest that HIV-1 has access to both $tRNA^{Met}$ for selection as the replication primer and supports a co-ordination between primer selection, translation and encapsidation during virus replication.

Background

A distinguishing feature of retrovirus replication is the process of reverse transcription in which the RNA genome is converted to a DNA intermediate prior to integration into the host cell chromosome. Reverse transcription is carried out by a virally encoded enzyme, reverse transcriptase [1, 2]. The initiation of reverse transcription occurs at a site near the 5' end of the viral RNA genome termed the primer-binding site (PBS) [3-5]. Initiation uses a host cell tRNA primer which is selected from the intracellular milieu and positioned at the PBS. Different retroviruses select specific tRNAs [6, 7]. For example, murine leukemia virus selects $tRNA^{Pro}$, avian leukosis virus selects $tRNA^{Trp}$ while lentiviruses, including human immunodeficiency virus type 1 (HIV-1), select $tRNA^{Lys,3}$ as the primer for reverse transcription [8-11].

The mechanism of tRNA primer selection by retroviruses is not completely understood. Studies with HIV-1 have suggested that interactions between Gag and Gag-

pol with host aminoacyl synthetase could facilitate the selection of $tRNA^{Lys,3}$ [12-14]. However, alteration of the PBS to be complementary to a number of different tRNAs allows these primers to be selected for reverse transcription [15-17]. Previous studies have shown that HIV-1 could stably utilize $tRNA^{His}$, $tRNA^{Glu}$, $tRNA^{Met}$, or $tRNA^{Lys1,2}$ if mutations of the PBS were accompanied by mutations within U5 to be complementary to the anticodon of the tRNA [18-23]. Recently, mutation of an additional region in U5, the primer activation site (PAS), to be complementary to $tRNA^{Lys1,2}$ has also been shown to allow continued selection of $tRNA^{Lys1,2}$ [24]. However, not all tRNAs can be stably used by HIV-1 as primers, even with A-loop modifications, suggesting that tRNA availability can influence preference for primer selection [22, 23].

Any understanding of tRNA primer selection needs to take into account the complex biosynthetic pathway of tRNAs and host cell translation. Following transcription in the nucleus, the tRNA interacts with a myriad of host cell proteins that are involved in processing, aminoacylation and transport from the nucleus to the cytoplasm [25]. The results of our previous studies have suggested a coupling between translation and selection of the tRNA primer used for reverse transcription [26]. In these studies, we found that tRNA transport from the nucleus to the cytoplasm was essential for selection and that aminoacylation of the tRNA, while not absolutely required, greatly enhanced the selection of the tRNA as a primer. Consistent with the link between primer selection and translation is that the synthesis of HIV-1 Gag is co-ordinated with encapsidation of genomic RNA [27].

In previous studies, we have described the construction and characterization of an HIV-1 in which the PBS was made complementary to $tRNA^{Met}$ used in translation

elongation ($tRNA^{Met(e)}$) [20, 22]. Upon extended culture of HIV-1 in SupT1 cells, the PBS reverted to utilize $tRNA^{Lys,3}$, although we were able to stabilize the use of $tRNA^{Met(e)}$ with additional mutations within U5. Two $tRNA^{Met}$ exist in cells that are involved in either initiation ($tRNA^{Met(i)}$) or elongation ($tRNA^{Met(e)}$) of translation [28, 29]. The tRNAs differ in eleven of the eighteen 3' terminal nucleotides (Figure 1) [28, 30] and interact with a different complement of host proteins that are involved in translational initiation or elongation [29]. Thus, HIV-1 with a PBS complementary to $tRNA^{Met(i)}$ or $tRNA^{Met(e)}$ would be expected to have to access different pools of $tRNA^{Met}$ and interact with different host cell proteins during primer selection. In the current study, HIV-1 in which the PBS was made complementary to $tRNA^{Met(e)}$ was shown to be replication competent and utilize this tRNA during early stages of *in vitro* culture prior to eventually reverting to utilize $tRNA^{Lys,3}$. In contrast, viruses in which the PBS were made complementary to $tRNA^{Met(i)}$, had reduced production of virus and were not infectious following long-term culture with SupT1 cells. Mutation of the AUG codon located in the PBS complementary to $tRNA^{Met(i)}$ restored infectivity of this virus but at levels lower than the wild type. Analysis of the PBS following replication revealed a preference for the PBS containing the mutated PBS (AUG to GUG). The results of these studies are discussed with respect to the co-ordination of HIV-1 primer selection, viral translation and encapsidation of the genomic RNA.

Results

Construction of HIV-1 proviruses with PBS complementary to $tRNA^{Met(e)}$ or $tRNA^{Met(i)}$

In previous studies, we have described the isolation and characterization of a HIV-1 mutant in which the PBS was complementary to $tRNA^{Met(e)}$ (HXB2-Met(e)) [20, 22, 31]. Subsequent characterization and re-derivation HXB2-Met(e) revealed that this virus could select $tRNA^{Met(e)}$ following short-term *in vitro* culture before reverting to utilize $tRNA^{Lys,3}$. For the current study, we constructed a HIV-1 proviral genome in which the PBS was made complementary to $tRNA^{Met(i)}$ [28, 30, 32]. The PBS of HXB2-Met(i) differs by 11 nucleotides from the PBS of HXB2-Met(e) (Figure 1).

Infectivity of HIV-1 with PBS complementary to $tRNA^{Met(e)}$ or $tRNA^{Met(i)}$

To characterize the effects of the PBS mutations on HIV-1 replication, we first analyzed the production of infectious virus following transfection of wild type and mutant proviral genomes. 293T cells were transfected with equal amounts of proviral DNA and the supernatants analyzed for the production of infectious virus using the JC53-BL assay [33]. The numbers of infectious units were calculated by determining the amount of cells expressing betagalactosidase following infection with culture supernatants. Modification of the PBS to be complementary to $tRNA^{Met(e)}$ (HXB2-Met(e)) resulted in production of infectious virus at approximately 20% level of the wild type virus. The reduced production of infectious virus as a result of alteration of the PBS has been found for viruses with different PBS [34, 35]. In contrast, viruses with the PBS complementary to $tRNA^{Met(i)}$ (HXB2-Met(i)) produced even lower amounts of infectious virus, approximately 2% of the wild type virus (Figure 2A). To further explore the nature

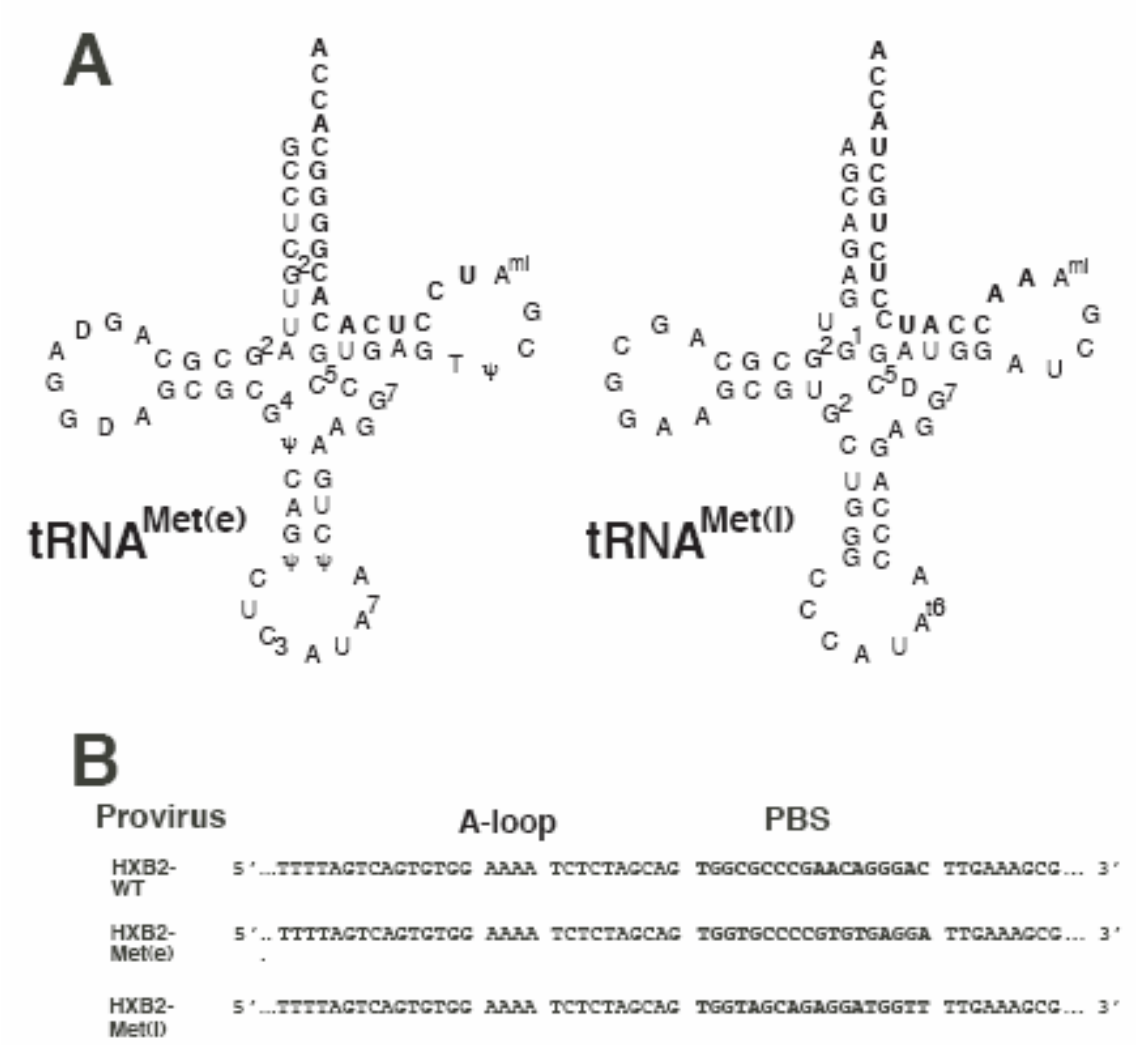

Figure 1. tRNA and HIV-1 proviruses.

Panel A. $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$. HeLa cell $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$. The nucleotides shown in large boldface type in the tRNA are complementary to the PBS of the viral RNA genome. Diagram of $tRNA^{Met}$ as described by Harada et al [49].

Panel B. Genomes with PBS complementary to $tRNA^{Met(e)}$ or $tRNA^{Met(i)}$. The 5' region of the HIV-1 RNA genome is expanded to depict the locations of sequences having complementarity with the 3' 18 nucleotides of the tRNA (bolded). The wild-type PBS (nucleotides 183 to 200) in HXB2 was replaced with the PBS complementary to the 3'-terminal 18 nucleotides of $tRNA^{Met(e)}$ [HXB2-Met(e)] or $tRNA^{Met(i)}$ [HXB2-Met(i)].

of the low production of infectious virus, we analyzed the culture supernatants for p24 antigen. Previous studies from this laboratory have demonstrated that viruses with alterations in the PBS produce similar levels of p24 antigen as wild type virus [15, 20, 21]. Consistent with these results, we found that transfecting a range of HXB2-Met(e) and HXB2-WT produced similar levels of p24 antigen in culture supernatants. In contrast, transfection HXB2-Met(i) yielded approximately 50% less p24 antigen in comparison to HXB2-Met(e) and HXB2-WT (Figure 2B). This reduced level of p24 antigen production of HXB2-Met(i) was consistent over a range of plasmid concentrations used for transfection. Thus, the alteration of the PBS to be complementary to $tRNA^{Met(i)}$ reduces the production of both infectious virus and p24 antigen in the culture supernatant.

One explanation for the reduced p24 could be that there is a disruption in the proteolytic processing of HIV-1, resulting in the production of greater levels of processed virions released from the cells. Since the p24 antigen ELISA does not efficiently recognize unprocessed Gag ($pr55^{Gag}$) this would result in lower amounts of virus detected from transfection of HXB2-Met(i). To address this issue, pelleted virus particles from culture supernatants were analyzed by Western blot with polyclonal antibodies against HIV-1 Gag (Figure 2C). HXB2-WT, HXB2-Met(e) and HXB2-Met(i) had greater CA p24 antigen than $pr55^{Gag}$, indicating that proteolytic processing was probably not effected by the alteration of the PBS. Interestingly, the Western blot revealed that the p24 antigen for HXB2-WT and HXB2-Met(e) was approximately 3 to 5 times that for $pr55^{Gag}$, whereas the ratio of CA p24 to $pr55^{Gag}$ for viruses derived from HXB2-Met(i) was considerably greater, approximately 10 to 50 times. Using recombinant $pr55^{Gag}$ as a

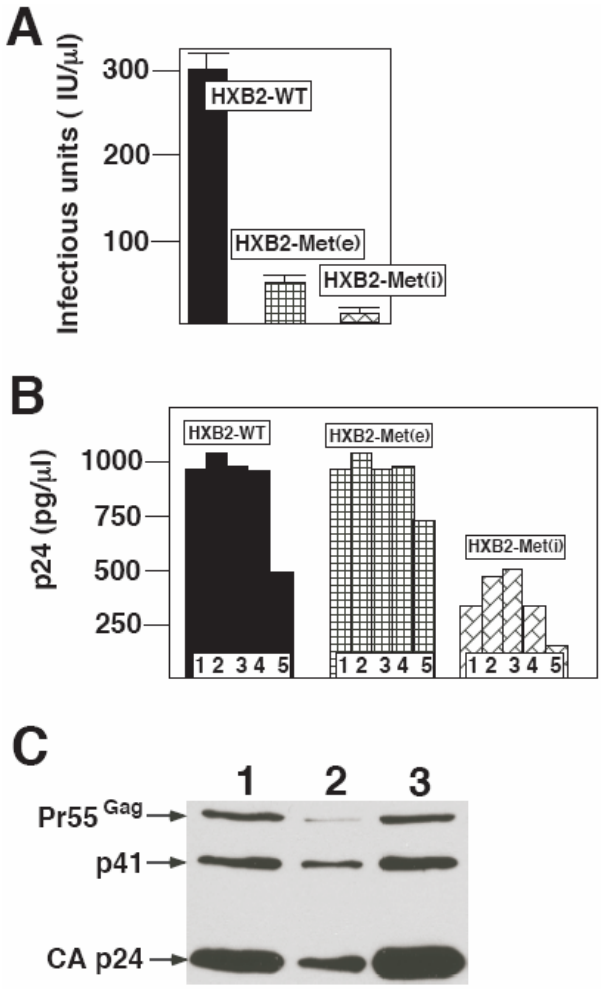

Figure 2. Characterization of recombinant viruses with PBS complementary to $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$.

Panel A. Production of infectious virus following transfection of proviral plasmids. The designated proviral plasmids were transfected into 293T cells and the supernatant assayed for production of infectious virus using the JC53-BL assay. Culture volumes for each virus were the same. Error bars $\pm$ standard deviation.

Panel B. p24 antigen production from transfected cells. Cells were transfected with different amounts of HXB2-WT, HXB2-Met(e) or HXB2-Met(i) and the p24 antigen in the culture supernatant was determined by solid phase ELISA. The amounts for each transfection was as follows: Lane 1 : 1 μg, Lane 2 : 2 μg, Lane 3 : 3μg, Lane 4 : 4 μg, Lane 5 : 8 μg of proviral plasmid DNA.

Panel C. Analysis of virus produced from transfected cells. Virus from transfected cells was pelleted by ultracentrifugation and subjected to SDS PAGE and Western blot using antibody specific for HIV-1 Gag. The order of the samples are as follows: Lane 1 – HXB2-Met(e), Lane 2 : HXB2-Met(i), Lane 3 : HXB2-WT. The positions of a viral *gag* gene products CA p24, p41 and $pr55^{Gag}$ are noted.

standard, we estimate that the levels of $pr55^{Gag}$ in viruses derived from HXB2-Met(i) was approximately 10 times less than that from viruses derived from the HXB2-Met(e) or HXB2-WT (data not shown). Collectively, the results of these studies demonstrate that alteration of the PBS to be complementary to $tRNA^{Met(i)}$, in contrast to viruses with a PBS complementary to $tRNA^{Met(e)}$, resulted in reduced production of virus particles.

Replication of HIV-1 with PBS complementary to $tRNA^{Met(e)}$ or $tRNA^{Met(i)}$.

We next examined replication of viruses in which the PBS was altered to be complementary to $tRNA^{Met(i)}$ or $tRNA^{Met(e)}$ in a continuous T cell line (SupT1). Although previous studies in this laboratory have shown that viruses with an altered PBS without mutations in the U5 region can utilize a variety of tRNA primers, a hallmark of all of these studies is the fact that the virus reverts to utilize $tRNA^{Lys,3}$ following *in vitro* culture [15-17]. As expected, wild type virus grew rapidly in these cultures resulting in many giant cell syncytia typical for HXB2-WT infection of SupT1 cells. Using the same amount of infectious virus, HXB2-Met(e) initially grew slower than HXB2-WT but eventually reached a level of p24 antigen in culture supernatants similar to that of wild type virus (Figure 3). Analysis of the PBS from integrated proviruses revealed that $tRNA^{Met(e)}$ was used as the primer for reverse transcription at early times of the infection but upon extended growth, the viruses reverted to utilize $tRNA^{Lys,3}$ (data not shown). In contrast, HXB2-Met(i) did not show detectable growth following infection of SupT1 cells. The p24 antigen amounts in culture supernatants did not increase over time and visually we did not observe giant cell syncytia indicative of a productive HIV-1 infection of SupT1 cells. We repeated this infection with 10 times the amount of p24 antigen, and

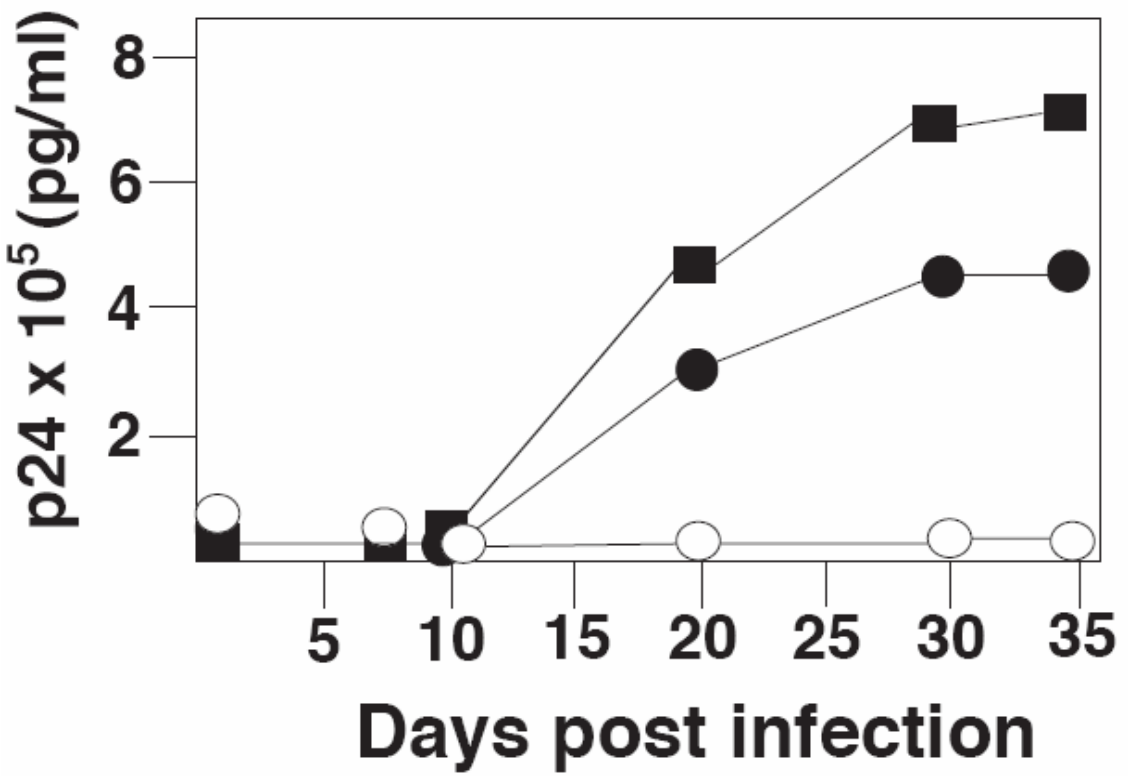

Figure 3. **Replication of virus with PBS complementary to $tRNA^{Met(e)}$ or $tRNA^{Met(i)}$.** Plasmids containing wild type or mutant proviral genomes were transfected into 293T cells. The virus was collected 48 hours later and the amount of infectivity determined using the JC53 assay. SupT1 cells were then infected with equal amounts of wild type or mutant viruses. The supernatant p24 measured at different times post infection. By day 21 and later, we recovered virus in which the PBS from HXB2-Met(e) had mutated to be complementary to $tRNA^{Lys,3}$. The culture for HXB2-Met(i) was extended to over 200 days with no subsequent rise in p24 antigen. Key: squares (HXB2-WT); closed circles (HXB2-Met(e)); open circles HXB2-Met(i).

again were unable to detect production of infectious virus following culture with SupT1 with HXB2-Met(i) (data not shown). In a third attempt, we increased the amount of HXB2-Met(i) so that the total amount of infectious virus was increased by 5 or 10 fold over the initial experiment. The amount used was equivalent to approximately 5 and 10 times the necessary infectious units needed by HXB2-WT or HXB2-Met(e) to initiate a productive infection. Even after extending the culture time to over 200 days, there was no evidence of infectious virus production as measured by p24 antigen capture (data not shown).

One explanation for the low infectivity of viruses with the PBS complementary to $tRNA^{Met(i)}$ is that the total amount of $tRNA^{Met(i)}$ in cells is limiting relative to $tRNA^{Met(e)}$. To address this issue, we compared the amounts of $tRNA^{Met(i)}$ with $tRNA^{Met(e)}$ and $tRNA^{Lys,3}$ found in SupT1 cells. We first established that our probes were specific for the individual tRNA species to be analyzed (data not shown). Using known amounts of *in vitro* transcribed tRNAs, we established a concentration curve to analyze the relative levels of the designated tRNAs (Figure 4). Under our experimental conditions, we found that the total amount of $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$ were comparable in SupT1 cells. The levels of $tRNA^{Met(e)}$ were approximately 50% that for $tRNA^{Lys,3}$. Similar amounts of $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$ were found in 293T cells (data not shown).

The reduced infectivity of HXB2-Met(i) could be a result of $tRNA^{Met(i)}$ not being selected from the intracellular milieu as the primer for HIV-1 reverse transcription. To address this issue, we analyzed the isolated viruses from transfection for the capacity to undergo minus strong-stop DNA synthesis (endogenous reaction). In this reaction, the viruses use the tRNA primer complementary to the PBS to initiate reverse transcription

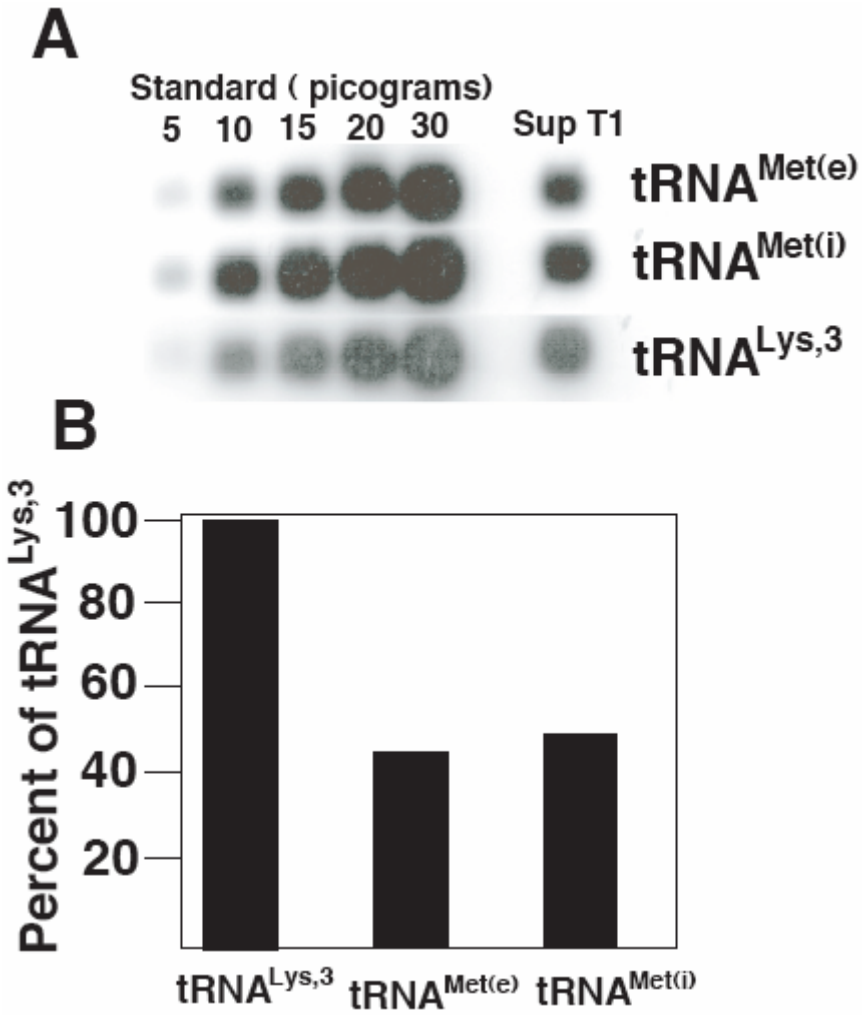

Figure 4. **Comparison of intracellular levels of $tRNA^{Met(e)}$ or $tRNA^{Met(i)}$.**
Panel A. Analysis of tRNAs from SupT1 cells. Increasing amounts of *in vitro* transcribed tRNA and total RNA isolated from SupT1 cells were subjected to Northern blot analysis. Each sample set was probed with the corresponding polynucleotide [γ-^{32}P] kinased oligo nucleotide. Shown is a picture of the audioradiogram from the probed samples. The amount of radioactivity in each spot was determined by excising the region and direct counting.
Panel B. Comparison of the relative amounts of tRNAs from SupT1 cells. The amounts of $tRNA^{Lys,3}$, $tRNA^{Met(e)}$ or $tRNA^{Met(i)}$ was determined from the quantitative analysis of the Northern blot presented in Panel A. When amount of $tRNA^{Lys,3}$ was set at 100%, the levels of $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$ were approximately equal and overall approximately 50% that of $tRNA^{Lys,3}$.

and synthesize minus strong-stop DNA. Previous studies from our laboratory have confirmed that the wild type virus uses $tRNA^{Lys,3}$ and the virus for HXB2-Met(e) uses $tRNA^{Met(e)}$ [20, 21]. Viruses were isolated from transfection supernatants by pelleting. The products from endogenous reverse transcription reactions were analyzed after different *in vitro* reaction times. The amounts of radionucleotide incorporation were then normalized to p24 levels (Figure 5). As the *in vitro* reaction time increased, we observed a linear increase in radioactivity from HXB2-WT. Similar observations were made for HXB2-Met(e) and HXB2-Met(i) albeit the levels that were approximately 70% those of the wild type virus. The amount of incorporation observed for HXB2-Met(e) were similar to those for HXB2-Met(i) when the values were normalized for p24 antigen (i.e. amount of virus particles). Collectively, the results of these studies suggest that both $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$ were able to be selected as the cognate tRNA as the primer for reverse transcription.

An AUG codon in the PBS of HXB2-Met(i) impacts production of virus

Finally, we noted that as a consequence of the alteration of the PBS to be complementary to $tRNA^{Met(i)}$, a new AUG sequence was present in the 5' NTR of the HIV-1 genome (Figure 6). In theory, this AUG could act to syphon off scanning ribosomes and reduce initiation of translation at the AUG used for synthesis of Gag. To address this possibility, we mutated the ATG to GTG in HXB2-Met(i) and determined the effect on the production of virus following transfection (Figure 6). The virus with A to G mutation (HXB2-Met(i)AG) produced similar amounts of virus as that from HXB2-WT or HXB2-Met(e) following transfection into 293T cells, consistent with the idea that

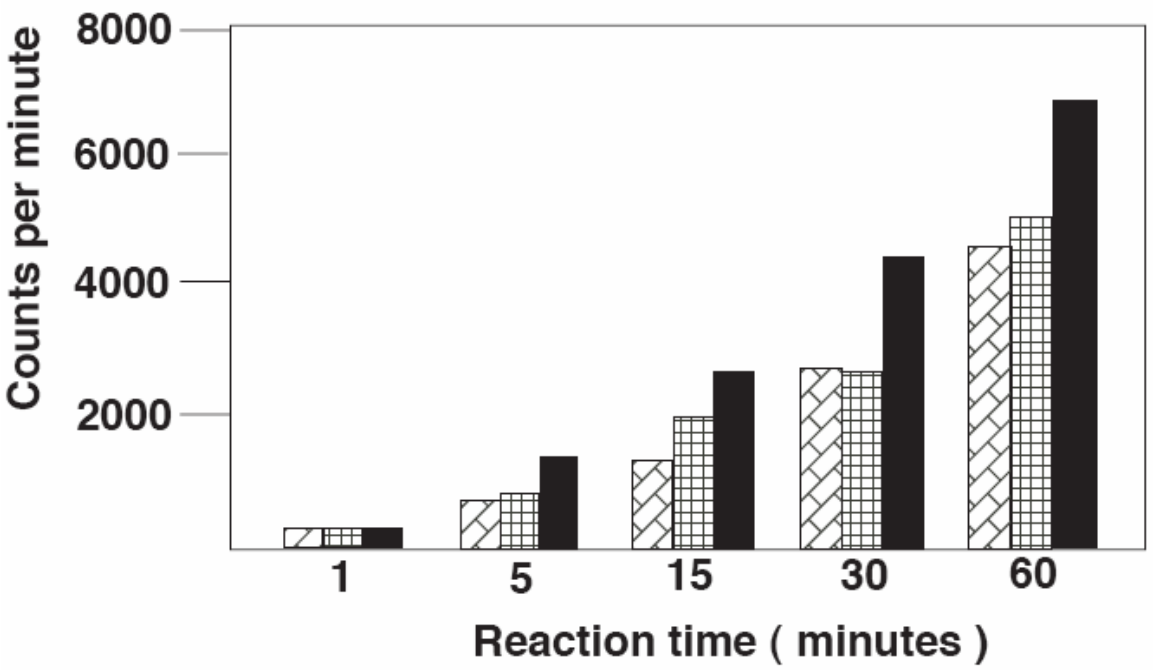

Figure 5. Endogenous reverse transcription of wild type and mutant viruses.
The endogenous reverse transcription assay was performed as described in the Materials and Methods. Autoradiography was used to identify radioactive areas, and the individual areas were excised and the radiation was quantitated using a scintillation counter. The values presented were then normalized to the levels of virus as determined by p24 antigen ELISA. The total reaction time was for 60 minutes with samples being assayed at 1, 5, 15, 30 and 60 minutes. The order of the samples are HXB2-Met(i) (rectangles), HXB2-Met(e) (squares) and HXB2-WT (solid bars). Data is representative from three independent experiments.

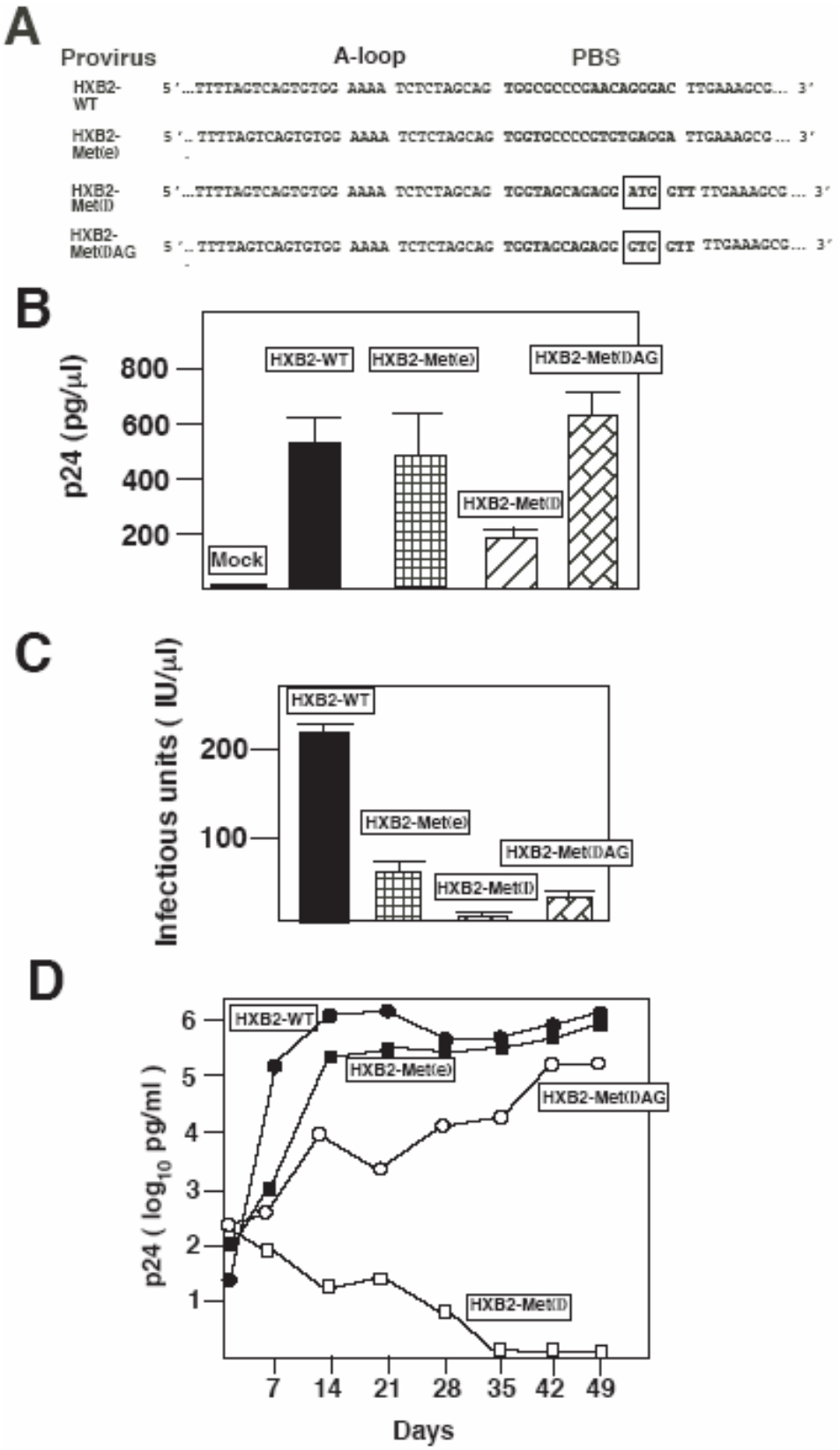
A
Provirus
A-loop
PBS
HXB2-WT 5'...TTTTAGTCAGTGTGG AAAA TCTCTAGCAG TGGCGCCCGAACAGGGAC TTGAAAGCG... 3'
HXB2-Met(e) 5'... TTTTAGTCAGTGTGG AAAA TCTCTAGCAG TGGTGCCCCGTGTGAGGA TTGAAAGCG... 3'
HXB2-Met(I) 5'...TTTTAGTCAGTGTGG AAAA TCTCTAGCAG TGGTAGCAGAGG ATC GTT TTGAAAGCG... 3'
HXB2-Met(I)AG 5'... TTTTAGTCAGTGTGG AAAA TCTCTAGCAG TGGTAGCAGAGG CTC GTT TTGAAAGCG... 3'
B
p24 (pg/μl)
800
600
400
200
Mock
HXB2-WT
HXB2-Met(e)
HXB2-Met(I)
HXB2-Met(I)AG
C
Infectious units (IU/μl)
200
100
HXB2-WT
HXB2-Met(e)
HXB2-Met(I)
HXB2-Met(I)AG
D
p24 (log10 pg/ml)
6
5
4
3
2
1
HXB2-WT
HXB2-Met(e)
HXB2-Met(I)AG
HXB2-Met(I)
7
14
21
28
35
42
49
Days

Figure 6. Analysis of HXB2-Met(i) with A to G mutation in PBS.
Panel A. HXB2-Met(i) with A to G mutation. The PBS of HXB2-Met(i) with the ATG codon as boxed. A new mutant, HXB2-Met(i)AG was constructed in which the adenine was changed to guanine to eliminate the ATG (boxed).
Panel B. Production of p24 following transfection. Proviral genome HXB2-Met(i)AG was transfected into 293T cells and the amount of virus produced was determined using the p24 antigen ELISA assay. For comparison, the p24 values for HXB2-Met(e), HXB2-Met(i) and HXB2-WT are presented. Error bars $\pm$ standard deviation.
Panel C. Production of infectious virus following transfection of proviral genomes into 293T cells. The amount of infectious virus is determined by the JC53-BL assay was determined for viruses derived from transfection of HXB2-Met(i)AG. For comparison, the amounts of infectious virus from HXB2-Met(e), HXB2-Met(i) and HXB2-WT are also presented. Error bars $\pm$ standard deviation.
Panel D. Replication of HXB2-Met(i) with A to G mutation in SupT1 cells. The replication of HXB2-Met(i)AG was analyzed in SupT1 cells. The amount of virus produced was determined by p24 antigen capture assay. Data is representative from two independent experiments. The samples are as marked in the figure.

elimination of the AUG restored the production of the virus. Analysis of the amount of infectious virus produced as measured by the JC53-BL assay revealed that lower amounts were produced than the wild type virus, but were now similar to that produced from transfection of HXB2-Met(e).

We next examined the replication of HIV-1 with the PBS complementary to $tRNA^{Met(i)}$ that contained the A to G mutation in SupT1 cells. Consistent with our previous result, HXB2-Met(i) did not demonstrate any significant increase in p24 antigen in the culture period (up to 49 days post initiation of culture). In contrast, HXB2-Met(i)AG had very low levels of replication up to Day 28, at which time virus levels slowly increased in the culture. Inspection of the cultures revealed the presence of syncytia, also confirming virus replication.

We next wanted to determine the status of the PBS in HXB2-Met(i)AG infected cultures. For these studies, we utilized PCR to amplify the U5-PBS region from integrated proviruses obtained at later times during culture when the virus replication was evident. Analysis of the U5-PBS from two different time points (Day 35 and Day 49) revealed presence of PBS complementary to $tRNA^{Met(i)}$ or PBS complementary to $tRNA^{Met(i)}$ with the A to G mutation ($tRNA^{Met(i)AG}$). In an earlier time point examined, we recovered approximately 50% of the TA clones from the PCR reaction were complementary to the $tRNA^{Met(i)}$ or $tRNA^{Met(i)AG}$. At the later time point, though, nearly all of the TA clones recovered (8 of 9) were complementary to the PBS with the A to G mutation (data not shown). Thus, HXB2-Met(i)AG had maintained the PBS complementary to $tRNA^{Met(i)}$ or $tRNA^{Met(i)AG}$ during replication and had not reverted back to utilize the wild type tRNA as was the case for HXB2-Met(e). Since the primer

selected for replication was $tRNA^{Met(i)}$, we expected the PBS with the A to G mutation would be converted back to complementarity with $tRNA^{Met(i)}$ and consequently the virus would gradually loose infectivity during the culture. The growth of the virus and the enrichment of viral genomes with the A to G change in the PBS at later culture times suggest that the viral genomes with the A to G change in the PBS were favored for encapsidation.

Discussion

Although the process of tRNA primer selection required for HIV-1 reverse transcription represents a critical step in replication, it is as yet unresolved as to how the virus is able to select tRNAs from the intracellular milieu that will subsequently be used in replication. HIV-1 has the capacity to utilize many different tRNA primers for replication, since alteration of the PBS corresponding to numerous tRNAs results in replication competent viruses [15-17]. The capacity to select many different tRNAs for primer selection suggests that this process mostly occurs at or near the site of translation, where the virus would have access to a variety of different tRNAs. To further explore a relationship between primer selection and translation, we wanted to determine if there were differences with respect to replication for HIV-1 viruses in which the PBS was complementary to $tRNA^{Met(e)}$ or $tRNA^{Met(i)}$. These tRNAs perform two different and distinct functions in the cell [28, 29]. Initiator tRNAs form a ternary complex with eukaryotic initiation factor 2 (eIF2) and GTP, which exclusively binds to the ribosomal P site and is excluded from the ribosomal A site. In contrast, $tRNA^{Met(e)}$ forms a complex with eEF1 and GTP and binds to the ribosomal A site [28, 29]. Thus, these two tRNAs

interact with different proteins and, quite possibly, are located within different micro-environments within the cytoplasm of the cell. If HIV-1 primer selection was co-ordinated with viral translation, we would expect that forcing the virus to use ($tRNA^{Met(i)}$) might impact on virus replication.

Transfection of HXB2-Met(e) and HXB2-WT produced similar amounts of virus, as measured by p24 antigen. Consistent with our previous results, HXB2-Met(e) was replication competent and grew to levels similar to that of wild type though, upon extended culture, these viruses did revert back to use $tRNA^{Lys,3}$ [20, 22]. It is important to note that the viruses used in this study did not contain the additional mutations within the U5 that are known to stabilize the virus to utilize $tRNA^{Met(e)}$ [20, 22]. In contrast, viruses in which the PBS was complementary to $tRNA^{Met(i)}$ were infectious, but at a level that was greatly reduced compared to HXB2-WT or HXB2-Met(e). Due to the low infectivity, the virus did not productively infect SupT1 cells. Since to date, this is the only HIV-1 with a PBS complementary to a mammalian tRNA that did not productively infect SupT1 cells, we further analyzed this virus to determine the reason for this phenotype. Characterization of this virus revealed that alteration of the PBS to be complementary to $tRNA^{Met(i)}$ resulted in a reduction in the overall amounts of virus (as measured by p24 antigen) and infectivity (as measured by the JC53-BL assay). The low infectivity of HXB2-Met(i) though was not due to overall lower levels of $tRNA^{Met(i)}$ compared to $tRNA^{Met(e)}$ in SupT1 cells. A previous study, also found that $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$ were present at similar levels in replicating cells, similar to the conditions seen in the continuously replications SupT1 cultures [36]. We also found that HIV-1 could select $tRNA^{Met(i)}$ for use as a primer. Using an endogenous reverse transcription reaction,

we found the levels of incorporation (representing minus strong stop DNA primed from the cognate tRNA) were similar for HXB2-Met(e) and HXB2-Met(i) following normalization to equal amounts of virus. The amount of endogenous reaction product for both HXB2-Met(i) and HXB2-Met(e) was less than that from HXB2-WT, consistent with the effect that alteration of the PBS has on infectivity. More importantly, the results demonstrate that there is no inherent problem with $tRNA^{Met(i)}$ that precludes its use as a primer for reverse transcription. There is, in fact, a precedence for $tRNA^{Met(i)}$ to be used as a primer for reverse transcription. Ty1 retrotransposons of yeast use $tRNA^{Met(i)}$ as the primer for transposition, which has many similarities with reverse transcription [37].

Most probably the major reason for the low replication of HXB2-Met(i) was the presence of an AUG in the 5' NTR prior to the start of Gag. From on our analysis, the AUG in the PBS of HXB2-Met(i) probably acted to syphon off scanning ribosomes, thus reducing the efficiency for start of Gag translation, resulting in the lower amount of virus production (and inability to sustain virus infection). Elimination of the AUG by the A to G mutation in the PBS restored virus production (by p24) and increased infectivity to levels similar to HXB2-Met(e). Virus replication was compromised though in SupT1 cells since $tRNA^{Met(i)}$ was still selected as the primer. The fact that HXB2-Met(i)AG replicated in SupT1 cells is consistent with our results that $tRNA^{Met(i)}$ can be selected and used as the primer, albeit at a lower efficiency than $tRNA^{Lys,3}$.

An unexpected result from our study occurred from the analysis of the PBS of HXB2-Met(i)AG following extended replication in SupT1 cells. Following reverse transcription, the PBS would be expected to contain fifty percent A to G mutations, inherited from RT copying the plus-strand RNA (generating minus-strand DNA), and

fifty percent PBS complementary to $tRNA^{Met(i)}$ plus-strand DNA inherited from copying the $tRNA^{Met(i)}$ primer. Unless a bias occurred during DNA repair, we would expect that from each completion of reverse transcription, the PBS of proviruses would contain equal numbers with and without the A to G mutation. Since, the proviruses with the PBS complementary to $tRNA^{Met(i)}$ would be non-infectious due to the AUG in the PBS, we would have expected that as a result of the continued use of $tRNA^{Met(i)}$, the numbers of repaired (A to G) PBS would be reduced following replication until no infectious virus was recovered. Surprisingly, we found that after extended culture time, the amount of virus increased, with the PBS containing the A to G change, suggesting that an additional selection occurred that favored the repaired genomes (A to G).

Conclusion

The results from our study suggest a link between primer selection, encapsidation of genomic RNA and translation. During translational elongation, microenvironments within the cytoplasm are probably created to facilitate translation. For example, a multi-component complex of aminoacyl synthetases have been characterized, forming what is referred to as a "nebula" of tRNA and host cell proteins (aminoacyl-synthetases, eEF-1) that facilitate efficient translation [38-40]. Possibly, HIV-1 primer selection has evolved to select the tRNA primer from a pool of tRNAs that are found in the micro-environment in the cytoplasm that occurs during translation. A previous study found that pseudovirions, composed of Gag and Gag-pol, contain similar amounts of $tRNA^{Lys,3}$ as the wild type virus, but pseudovirions composed of Gag without Gag-pol do not show enhanced $tRNA^{Lys,3}$ incorporation [41, 42], suggesting that Gag-pol could be involved

with primer selection. It is also possible that the translation of Gag-pol could facilitate primer selection. This process could also be co-ordinated with the interaction of Gag with the genomic RNA, which has been suggested as a signal for encapsidation [27, 43-45]. Gag binding to the 5' non-translated region of the HIV-1 genome would also inhibit new translation. This process could be co-ordinated, or facilitated by the tRNA interaction with the PBS and targeting the genomic RNA for encapsidation. Targeting of genomic RNAs that are used in translation for encapsidation would explain the capacity of HXB2-Met(i)AG to amplify in culture even though $tRNA^{Met(i)}$ was still selected as the primer for reverse transcription. Additional studies will be required to test this possibility and to further delineate the link between primer selection, encapsidation and translation of genomic RNA.

Materials and Methods

Tissue culture

The 293T and JC53BL cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Cellgro by Mediatech, Inc.), L-glutamine, antibiotics and 10% fetal bovine serum. The SupT1 cells were maintained in RPMI 1640, L-glutamine, antibiotics and 15% FBS.

HIV-1 Proviral Mutants

The PBS of the provirus HXB2 was mutated to be complimentary to the 3' end of the human tRNA elongator methionine ($tRNA^{Met(e)}$) to create (HXB2-Met(e)) [20, 22, 46]. To construct an HIV-1 with a PBS complementary to $tRNA^{Met(i)}$ as well as to

$tRNA^{Met(i)}$ with an A to G mutation at position 13 ($tRNA^{Met(i)AG}$), the Quickchange II Site Directed mutagenesis Kit (Stratagene) was used with the following sets of primers:

5' ggaaaatctctagcagtggtagcagaggatggttctgaaagcgaaagggaaac 3' Met(i)

5' gtttccctttcgctttcagaaccatcctctgctaccactgctagagattttcc 3'

5' ggaaaatctctagcagtggtagcagagggtggttctgaaagcgaaagggaaac 3' Met(i) AG

5' gtttccctttcgctttcagaaccaccctctgctaccactgctagagattttcc 3'

and the transfer plasmid pUC119 HXB2 as previously described (the underlined nucleotides correspond to the mutation to create PBS complementary to $tRNA^{Met(i)}$ or $tRNA^{Met(i)AG}$, respectively. [20, 22]. *HpaI* and *BssHII* (New England Biolabs) were used to excise an 868 base pair fragment from the transfer plasmid and HXB2-WT. The products of the digestion reaction were run on a 1% agarose gel. Fragments containing the PBS mutation as well as the HXB2-WT backbone, were excised and gel purified (Qiagen). Finally the fragments were ligated into the HXB2-WT backbone. The resulting plasmids were named HXB2-Met(i) and HXB2-Met(i)AG, respectively, and are isogenic with HXB2 except for the PBS.

DNA Transfections

Transfections were performed according to the protocol for the Fugene 6 transfection reagent (Roche Molecular Biochemicals). Briefly, proviral plasmid DNA and Fugene reagent (varying amounts depending upon the experiment) were added to 100 microliters of DMEM without FBS. This mixture was incubated at room temperature for approximately 45 minutes and added drop wise to one well of a six well plate containing 60% of 293T cells and DMEM and 10% FBS. The transfections were incubated

overnight at 37^{o} and the medium was then replaced with fresh DMEM containing 10% FBS. After 48 hours, all the supernatants were collected, filtered and stored at –80^{o} C. The supernatants from the transfected cells were assayed for HIV-1 p24 (Beckman Coulter) and the infectivity was determined using the JC53-BL assay [33].

Analysis of virus replication

1 x 10^{6} SupT1 cells were infected with equal amounts of infectious virus (200IU) as measured by the JC53 reporter assay. The infected SupT1 cells were monitored visually for the formation of syntitia and cell counts were maintained at 1 x 10^{6} cells/ml at time of passage either by removing or adding additional cells. 1.5 ml of SupT1 cultures were collected at various time points. The cultures were spun down in a table-top centrifuge at 13000xg for 1 minute. Supernatant was removed and analyzed for p24 antigen by ELISA (Coulter Laboratories).

PCR amplification and DNA sequencing of PBS-containing proviral DNA

Following the manufacturer's instructions, high molecular DNA was isolated from the cells that remained after centrifugation of cultures and removal of supernatant using a Wizard Genomic DNA Isolation Purification Kit (Promega). Cellular DNA was used to amplify the U5 and PBS regions of integrated proviral DNA sequences by using the following HIV-1 proviral-DNA-specific primers: 5' cggaattctctccttctagcctccgctagt 3' and 5' ccttgacgatgcgatctaccacacacaaggc 3'. PCR-amplified DNA was directly ligated into the pGEM-T-easy vector (Promega). Following transformation into *E. coli* and

screening, the U5-PBS-containing plasmid DNAs prepared from individual recombinant clones were sequenced by using the primer 5' cggaattctctccttctagcctccgctagt 3'.

RNA Isolation and analysis of tRNA

Tri Reagent™ (Sigma Chemicals) was used to extract total cellular RNA from SupT1 cells as previously described [46]. *In vitro* transcribed tRNA standards for $tRNA^{Lys,3}$, elongator methionine ($tRNA^{Met(e)}$) and initiator methionine ($tRNA^{Met(i)}$), cDNA with T7 promoters upstream were constructed via PCR of the following pairs of overlapping primers, respectively. Capital letters denote sequence for T7 promoter:

1. 5'aattTAATACGACTCACTATAGGcccggatagctcagtcgg3' and 5'cgcccgaacagggacttgaaccctggaccctcagattaaaagtctgatgctctaccgactgagctatccgggc 3' ($tRNA^{Lys,3}$);

2. 5'aattTAATACGACTCACTATAGGcctcgttagcgcagtagg3' and 5'tgccccgtgtgaggatcgaactcacgaccttcagattatgagactgacgcgctacctactgcgctaacgagg 3' ($tRNA^{Met(e)}$);

3. 5'aattTAATACGACTCACTATAGGagcagagtggcgcagcgg3' and 5'tagcagaggatggtttcgatccatcgacctctgggttatgggcccagcacgcttccgctgcgccactctgct 3' ($tRNA^{Met(i)}$);

The tRNAs were synthesized from the cDNA using the T7 – Megashortscript Transcription Kit (Ambion) and purified from agarose gel as previously described [47]. In order to determine the relative amounts of tRNA present in SupT1 cells, total cellular tRNA and various amounts of synthesized tRNA as standards were separated by electrophoresis and blotted using NorthernMax™-Gly Kit (Ambion) [47]. The 3'

primers used in the synthesis of the tRNA cDNA were kinased with [□-^{32}P] ATP and used as probes and exposed to X-ray film as previously described [47]. Radioactivity was quantitated by excising the product and directly counting (Cherenkov). A plot of the amount of the radioactivity versus amount of *in vitro* transcribed tRNA was used to derive a standard curve to quantitate the tRNA amounts from SupT1 cells. All values obtained for each tRNA were within the linear range of the standard curve.

Endogenous Reverse Transcription Reaction

Large scale transfections of 293T cells with HXB2-WT, HXB2-Met(e) and HXB2-Met(i) with Fugene 6 were done and supernatant was collected after 72 hours. Virus was concentrated by ultracentrifugation at 27000 rpm overnight by an SW28 rotor and the pellet was resuspended in about 200µl Tris pH 8.2. For the endogenous RT reaction, Tris pH 8.0 (67 mM), DTT (67 mM), $MgCl_2$ 200 mM, KCL (6.6 mM) Triton X-100 (0.1%) and EDTA (0.66 mM) with 0.5 mM, dATP, dCTP, dGTP (Amersham) were added with 50 µCi of [∝-^{32}P]-dTTP. For each condition, 15µl of resuspended viral pellet was placed in a well of a 96 well round-bottom plate and 75µl of reaction cocktail was added. The RT reaction was carried out at 37^{o}C for 1, 5, 15, 30, 60 minutes and stopped adding 50µl 0.2M $Na_4P_2O_7$. The samples were then transferred to a biodyne B membrane N/Str PS 0.45µm pore size (Nalge Nunc International). Using a vacuum manifold, the samples were aspirated through the membrane with 150µl buffer (NaH_2PO_4, dH_2O pH 6.8) was passed through the membrane twice as a wash. Further washing was accomplished by placing the membrane into wash buffer and onto an orbital shaker for 10-15 minutes. The membrane was dried and exposed for 4 hours at –80^{o}C.

The individual wells were cut out and radiation was quantitated using a scintillation counter.

SDS-Page and Immunoblotting

10μl of the pelleted virus was analyzed by SDS-polyacrylamide gel (12%) electrophoresis (SDS-PAGE). Following SDS-PAGE, a Trans-Blot (Biorad) was used to transfer proteins to NitroPure nitrocellulose membrane (Micron Separations, Westborough, Mass.). Membranes were blocked in 2% fat free milk overnight. The membrane was washed with TBS-Tween 20 for 30 minutes and then incubated with the primary Rabbit anti HIV-1 p24 polyclonal antibody (NIH AIDS Repository, Catalog # 4250) suspended in 2% milk for 60 minutes, washed several times with TBS-Tween and incubated with mouse anti-rabbit Ab conjugated with horseradish peroxidase in 2% milk/TBS for 60 minutes. Following washing with several changes, the blot was incubated in ECL detection reagent according to the manufacture's directions (Amersham). Membranes were then exposed to X-ray film and were scanned; the intensity of the bands analyzed using ImageJ [48].

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SELECTION PREFERENCE FOR THE HIV-1 PRIMER tRNA USED IN REVERSE TRANSCRIPTION IS CONTROLLED BY INTRACELLULAR AVAILABILITY AND THE INTERACTION WITH THE U5-PBS

by

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Abstract

Human immunodeficiency virus (HIV-1) uses $tRNA^{Lys,3}$ as the primer for reverse transcription. HIV-1 can be forced to use $tRNA^{Lys1,2}$ if the primer binding site (PBS) and the A-rich region just upstream of the PBS are altered to be complementary to the 3' terminal 18-nucleotides and the anticodon of $tRNA^{Lys1,2}$, respectively. However, the virus that is forced to use $tRNA^{Lys1,2}$ is less infectious than wild type virus in human peripheral blood mononuclear cells (PBMC). In a recent study, we have found that alteration of the codons prior to the Gag-pol frameshift region so as to be specific for $tRNA^{Lys1,2}$ improves the replication of HIV-1 forced to use $tRNA^{Lys1,2}$ as the primer, suggesting a link between viral translation and primer selection. To determine the effects of the A-loop and PBS regions have on the capacity of HIV-1 with altered codon usage in Gag-pol to grow in PBMCs, viruses were created in which the PBS alone was made complementary to $tRNA^{Lys1,2}$ with and without enrichment of the codons of the Gag-pol frameshift region specific for $tRNA^{Lys,2}$. These viruses replicated similar to wild type, but failed to maintain the PBS complementary to $tRNA^{Lys1,2}$ and reverted to utilize $tRNA^{Lys,3}$ following replication. Viruses in which the A-loop region was altered to be complementary to $tRNA^{Lys1,2}$ with a PBS complementary to $tRNA^{Lys,3}$ (wild type) maintained a PBS complementary to $tRNA^{Lys,3}$ following replication in PBMCs, although they were less infectious than the wild type virus regardless of whether the additional mutation in the Gag-pol frameshift region was present. Finally, we analyzed the effects of forcing HIV-1 to utilize $tRNA^{Met}$ as the primer for reverse transcription. Viruses with a PBS complementary to $tRNA^{Met}$ and A-loop complementary to $tRNA^{Lys1,2}$, with and without the lysine codon change in Gag-pol, had severely compromised replication

compared to the wild type virus but maintained a PBS complementary to $tRNA^{Met}$ following *in vitro* replication in PBMCs. The results of these studies demonstrate that amino acids prior to the Gag-pol frameshift region can influence virus replication, but the control for the specificity of primer selection resides with the 5' end of the viral genome containing the A-loop region and the PBS.

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 protein reverse transcriptase (Baltimore, 1970; Temin and Mizutani, 1970). In order for the reverse transcriptase to copy the viral RNA genome, a primer is needed. Retroviruses have evolved to select host tRNAs as the primer for reverse transcription (Mak et al., 1997; Marquet et al., 1995). All lentiviruses, including human immunodeficiency virus type 1 (HIV-1) select $tRNA^{Lys,3}$ as the primer for initiation of reverse transcription (Mak et al., 1997; Marquet et al., 1995). How and why HIV-1 selects $tRNA^{Lys,3}$ as the primer for initiation of reverse transcription is unknown. The 3' terminal 18-nucleotides of the tRNA selected as the primer is complementary to a region on the viral genome known as the primer-binding site (PBS) (Gilboa et al., 1979; Varmus, 1988; Varmus, 1982). Previous studies have found that alteration of the PBS of HIV-1 to be complementary to alternative tRNAs (e.g. $tRNA^{Lys,3}$) results in the capacity of HIV to utilize these tRNAs transiently for *in vitro* replication (Das, Klaver, and Berkhout, 1995; Li et al., 1994; Wakefield, Wolf, and Morrow, 1995). Ultimately though, all of these viral genomes

revert back to utilize $tRNA^{Lys,3}$ following limited *in vitro* culture. Thus HIV, and other lentiviruses, have evolved a mechanism to insure that $tRNA^{Lys,3}$ will be the preferred tRNA for selection as the primer for reverse transcription.

Early studies demonstrated that $tRNA^{Lys,3}$ is enriched in HIV-1 virions (Jiang et al., 1994; Zhang et al., 1996). In contrast to pseudovirions which contain only Gag, pseudovirions that contain Gag-pol were enriched for both $tRNA^{Lys,3}$ and $tRNA^{Lys1,2}$ (Jiang et al., 1993; Mak et al., 1994). Further studies have revealed that lysyl-synthetase was also incorporated into the virion; the lysl-synthetase interacts with Gag giving rise to the suggestion that this protein could act as a chaperone for $tRNA^{Lys,3}$ (Cen et al., 2002; Javanbakht et al., 2003). Studies from this laboratory have used an approach in which mutations within the PBS were altered to determine if alternative tRNAs could be stably selected as primers for initiation of reverse transcription. Numerous tRNAs were examined for the capacity to be utilized by HIV-1 as the primer for reverse transcription. With only one exception (virus with a PBS complementary to $tRNA^{Met}$), viruses with the PBS altered to be complementary to tRNAs other than $tRNA^{Lys,3}$ reverted back to utilize $tRNA^{Lys,3}$ following limited *in vitro* replication (Dupuy et al., 2003; Kang and Morrow, 1999; Kang, Zhang, and Morrow, 1997; Kang, Zhang, and Morrow, 1999; Wakefield, Kang, and Morrow, 1996; Zhang et al., 1998). Even viruses in which the PBS was mutated to be complementary to $tRNA^{Lys1,2}$, which is found within HIV-1 virions, reverted to use $tRNA^{Lys,3}$ following *in vitro* replication (Jiang et al., 1994; Zhang et al., 1996). However, viruses in which the PBS was complementary to $tRNA^{Met}$ stably maintained the PBS following replication even though $tRNA^{Met}$ was not found in substantial quantities in HIV-1 virions (Moore-Rigdon et al., 2005; Wei et al., 2005).

Thus, the capacity of tRNA to be incorporated into HIV-1 virions does not necessarily explain the preferential use of $tRNA^{Lys,3}$ as the primer for reverse transcription.

Previous studies have utilized both chemical and enzymatic analysis to investigate the initiation complex formed between $tRNA^{Lys,3}$ and the HIV-1 proviral genome (Isel et al., 1995; Isel et al., 1993). In addition to interactions between the 3' terminal 18-nucleotides of the tRNA and the PBS, a second interaction was found between the anticodon region of $tRNA^{Lys,3}$ and a region upstream of the PBS designated as the A-loop. Previous studies from this laboratory have demonstrated that a mutation within the A-loop to be complementary to the anti-codon of certain tRNAs (including $tRNA^{Lys1,2}$), in conjunction with alteration of the PBS, allows HIV-1 to stably utilize these tRNAs for replication (Dupuy et al., 2003; Kang and Morrow, 1999; Kang, Zhang, and Morrow, 1997; Kang, Zhang, and Morrow, 1999; Ni and Morrow, 2006; Wakefield, Kang, and Morrow, 1996; Zhang et al., 1998). These viruses replicate at lower levels than the wild type virus (Kerr and Sharpe, 1989). Even with A-loop modifications though, not all tRNAs can be stably selected and used by HIV-1 for reverse transcription. Characterization of HIV-1 in which the A-loop and PBS were mutated to be complementary to the anticodon and 3' terminal nucleotides of $tRNA^{Lys1,2}$ revealed that this virus can stably utilize this tRNA for replication, but is less infectious than the wild type virus which utilizes $tRNA^{Lys,3}$ (Kang, Zhang, and Morrow, 1999; Moore et al., 2004). This highlights the fact that even though tRNAs can interact with lysl-synthetase (as do $tRNA^{Lys,3}$ and $tRNA^{Lys1,2}$), it does not necessarily mean that HIV-1 will prefer $tRNA^{Lys1,2}$ over $tRNA^{Lys,3}$, suggesting that other aspects of virus replication are important for primer selection. In recent studies, we have further addressed a reason for the

discrepancy between the preference for $tRNA^{Lys1,2}$ and $tRNA^{Lys,3}$. Previous work from our laboratory has suggested that the process of primer selection is coupled with viral translation (Kelly, Palmer, and Morrow, 2003). We noted that the tRNAs that could be stably selected and used by HIV-1 for replication had codons that were enriched prior to the Gag-pol frameshifting site; in contrast, corresponding codons of the non-preferred were not found near the Gag-pol frameshift site, leading to the postulation that the process of frameshifting to synthesize the Gag-pol polyprotein could be linked with primer selection [Palmer, Submitted to J. Virol.]. To support this hypothesis, we demonstrated that viruses with the A-loop and PBS complementary to $tRNA^{Lys1,2}$ would show enhanced replication if codons near the Gag-pol frameshift site were mutated so as to utilize $tRNA^{Lys1,2}$. In the current study, we have extended this work to further explore the role of the A-loop and PBS in the selection process. Viruses were generated which contain mutations within the frameshift site to enrich for $tRNA^{Lys1,2}$ with PBS and A-loop complementarity to either $tRNA^{Lys,3}$ or $tRNA^{Lys1,2}$. In addition, we utilized a third virus in which the PBS was altered to be complementary to $tRNA^{Met}$. Previous studies from our laboratory have shown that this tRNA can be stably selected by HIV-1 for virus replication, although the level of virus replication is impaired in comparison to wild type (Moore-Rigdon et al., 2005). We wanted to determine if the primer preference for $tRNA^{Met}$ could be influenced by codon changes to favor $tRNA^{Lys1,2}$ or $tRNA^{Lys,3}$. The results from our analysis with viruses designed to utilize $tRNA^{Met}$, $tRNA^{Lys1,2}$ or $tRNA^{Lys,3}$ reveals that tRNA complementarity with the PBS is the most important determinant for primer selection. Overall, our studies provide new insights into the mechanism of HIV-1 primer selection and the preference of HIV-1 for $tRNA^{Lys,3}$.

Results

HIV-1 proviral genomes with mutated A-loop regions and PBS complementary to tRNA^{Lys1,2}

In previous studies, we have described the construction and characterization of HIV-1 genomes in which the A-loop and PBS were mutated to be complementary to the anticodon and 3' terminal nucleotides of tRNA^{Lys1,2} (Kang, Zhang, and Morrow, 1999; Moore et al., 2004). Characterization of this virus revealed that it stably maintained the PBS complementary to tRNA^{Lys1,2} but replicated at levels that were considerably less than that for the wild type (Moore et al., 2004). In a recent study, we have further modified this virus such that the 5 codons for lysine prior to the Gag-pol frameshift site were mutated to be specific for tRNA^{Lys1,2} and have shown that this significantly enhances the infectivity of this virus compared to that of the same virus with the wild type codons [Palmer, Submitted to J. Virol.]. To determine the effects of A-loop region modification on the replication and stability of the virus, we have made additional mutations in which the A-loop regions were re-constructed to be complementary to tRNA^{Lys,3}, while the PBS remained complementary to tRNA^{Lys1,2} (Figure 1).

Viruses with both the wild type and codon mutations within the frameshift were then analyzed for growth and stability of the PBS. To generate viruses, proviral plasmids were transfected in the 293T cells. The resulting virus was then analyzed for infectivity using the JC53-BL assay (Moore et al., 2004). Infection of human peripheral blood mononuclear cells (PBMCs) that had been previously activated in the presence of IL-2, were initiated using equal amounts of infectious virus. The amount of infectious virus produced was then determined using the JC53-BL assay. Similar to results obtained in

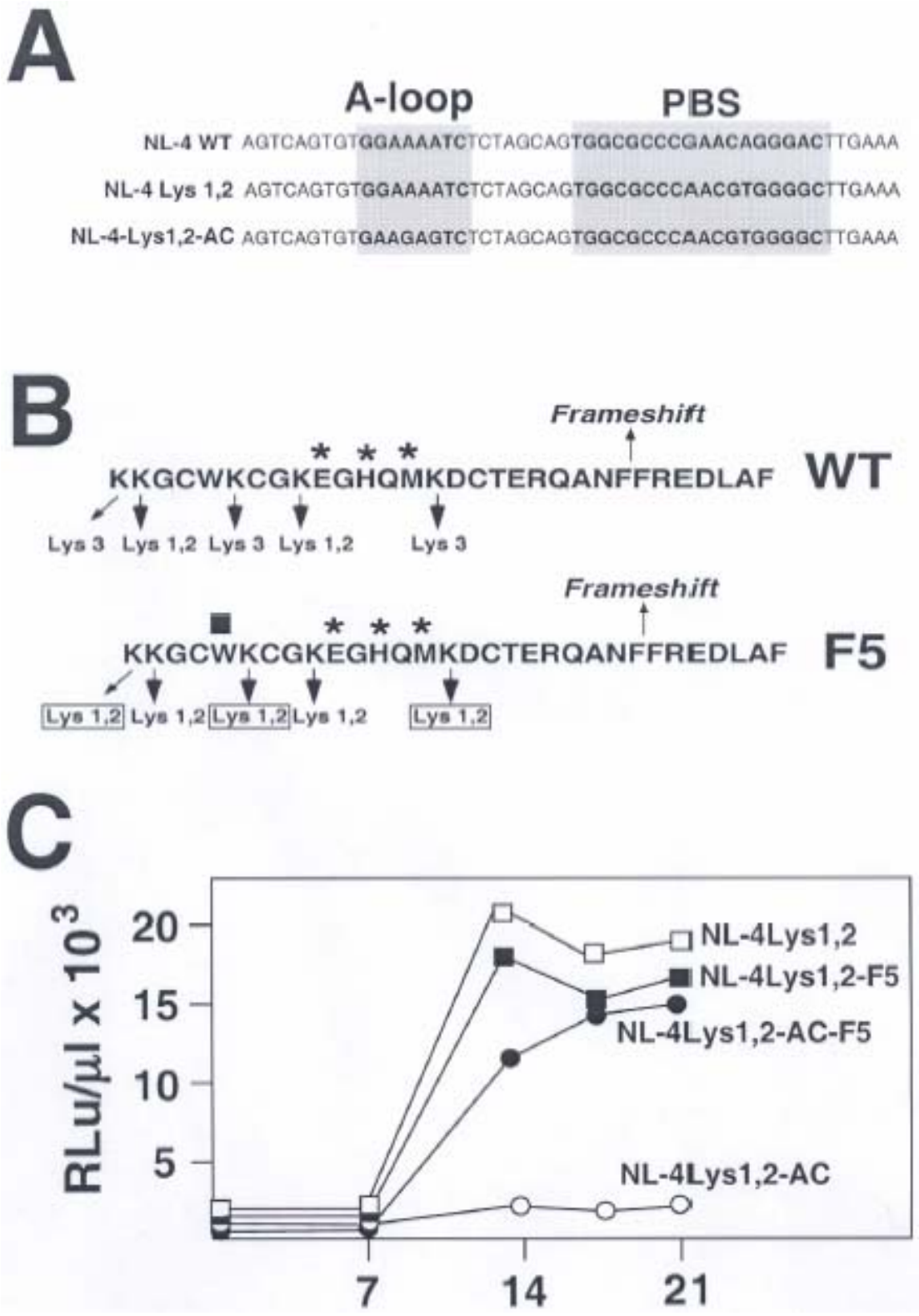
A
A-loop
PBS
NL-4 WT AGTCAGTGTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACTTGAAA
NL-4 Lys 1,2 AGTCAGTGTGGAAAATCTCTAGCAGTGGCGCCCAACGTGGGGCTTGAAA
NL-4-Lys1,2-AC AGTCAGTGTGAAGAGTCTCTAGCAGTGGCGCCCAACGTGGGGCTTGAAA
B
Frameshift
KKGCWKCGKEGHQMKDCTERQANFFREDLAF
WT
Lys 3 Lys 1,2 Lys 3 Lys 1,2 Lys 3
Frameshift
KKGCWKCGKEGHQMKDCTERQANFFREDLAF
F5
Lys 1,2 Lys 1,2 Lys 1,2 Lys 1,2 Lys 1,2
C
RLu/µl x 10^3
20
15
10
5
7
14
21
NL-4Lys1,2
NL-4Lys1,2-F5
NL-4Lys1,2-AC-F5
NL-4Lys1,2-AC

Figure 1. HIV-1 genome with mutations in A-loop-PBS and codons prior to Gag-pol.

Panel A. HIV-1 genomes with mutations in the U5-PBS. The U5-PBS of the wild type genome (NL4) was modified to be complementary to the anticodon and 3' terminal nucleotides of $tRNA^{Lys1,2}$ (shaded). The resulting plasmid, NL4-Lys12-AC has been previously described (Kang, Zhang, and Morrow, 1999).

Panel B. Modification of the codons prior to the Gag-pol frameshift site to be specific for $tRNA^{Lys1,2}$. In a previous study, we have shown that modification of the codons for the 5 lysine amino acids prior to the Gag-pol frameshift region can affect the replication of viruses designed to utilize $tRNA^{Lys1,2}$. Depicted is the modification resulting in the HIV-1 proviral genome with codons specific for $tRNA^{Lys}$. Proviral plasmids were constructed with the wild type U5-PBS (NL4) or the A-loop PBS modified to allow use of $tRNA^{Lys1,2}$ (NL4-Lys12-AC) with both the wild type Gag-pol frameshift region (WT) or the mutation with the codon specific for $tRNA^{Lys1,2}$ (F5).

Panel C. Replication of HIV-1 with A-loop-PBS complementary to $tRNA^{Lys1,2}$. The replication of viruses with a wild type A-loop-PBS or PBS complementary to $tRNA^{Lys1,2}$ were analyzed in PBMC. The virus replication was monitored over a 28-day *in vitro* culture; additional PHA activated PBMC were added at Day 14. At the indicated sampling times, supernatants were assayed for infectious virus using the JC53-BL assay. Data is representative of two independent experiments.

our previous study, we found that viruses with the PBS and A-loop complementary to $tRNA^{Lys1,2}$ were infectious but replicated at a level that was significantly lower than that for viruses without the lysine codons near the Gag-pol frameshift region specific for $tRNA^{Lys1,2}$ (F5). In contrast, virus in which the A-loop was altered to be complementary to $tRNA^{Lys,3}$ (wild type) with a PBS still complementary to $tRNA^{Lys1,2}$ resulted in enhanced replication compared to viruses with the A-loop complementary to $tRNA^{Lys1,2}$.

We next determined the stability of the PBS following *in vitro* replication. For these studies, PCR was used to amplify the PBS region obtained from integrated proviruses at the later times of infection. Consistent with our previous studies, viruses with the A-loop and PBS complementary to $tRNA^{Lys1,2}$ stably maintained a PBS complementary to $tRNA^{Lys1,2}$ throughout the infection period. In contrast, analysis of the PBS region from viruses in which the A-loop had been altered to be complementary to the $tRNA^{Lys,3}$ (wild type) all reverted to utilize $tRNA^{Lys,3}$ following replication (data not shown). Additional mutations within the codons prior to the Gag-pol frameshift region though did not prevent the reversion to utilize $tRNA^{Lys,3}$. The results of these studies then demonstrate the strict requirement for the A-loop modification to maintain the PBS complementary to $tRNA^{Lys1,2}$.

Effect of A-loop modification on replication of wild type HIV-1

Previous studies from our laboratory have suggested that modifications of the A-loop can impact on viral replication (Dupuy et al., 2003; Kang, Zhang, and Morrow, 1999; Wakefield, Kang, and Morrow, 1996; Zhang et al., 1998). To test the effects of the codon mutations upstream of the Gag-pol frameshift site favoring $tRNA^{Lys1,2}$ usage, we

constructed HIV-1 proviral genomes in which the A-loop was modified to be complementary to $tRNA^{Lys1,2}$ (Figure 2). Viruses were generated following transfected into 293T cells; the amount of infectious virus was determined by the JC53-BL assay and equal amounts were used to initiate infections in human PBMC. The alteration of the A-loop resulted in a delay in the replication of both viruses. The peak replication for both viruses was less than that for the wild type virus with or without the additional modification in the codons of the Gag-pol frameshift site. Although alteration of the A-loop region effected the production of infectious virus, it did not result in these viruses utilizing $tRNA^{Lys1,2}$ as the primer for replication. Analysis of the PBS of integrated proviruses revealed that all maintained a PBS complementary to $tRNA^{Lys,3}$ (data not shown).

Analysis of replication of virus with a PBS complementary to $tRNA^{Met}$

Previous studies from our laboratory have described the characterization of HIV-1 in which the PBS has been mutated to be complementary to $tRNA^{Met}$ (Moore-Rigdon et al., 2005). During the process of characterization, we noted that under certain circumstances this virus would convert to utilize $tRNA^{Lys1,2}$ (Kang and Morrow, 1999; Kang, Zhang, and Morrow, 1999). In the current set of experiments, we attempted to facilitate this conversion through construction of HIV-1 proviral genomes in which the A-loop had been mutated to be complementary to $tRNA^{Lys1,2}$ while the PBS was complementary to $tRNA^{Met}$. We also constructed viruses in which the amino acids prior to the Gag-pol frameshift were mutated to favor the use of $tRNA^{Lys1,2}$. Viruses were generated by transfection of proviral genomes into 293T cells and the amount of infectious virus

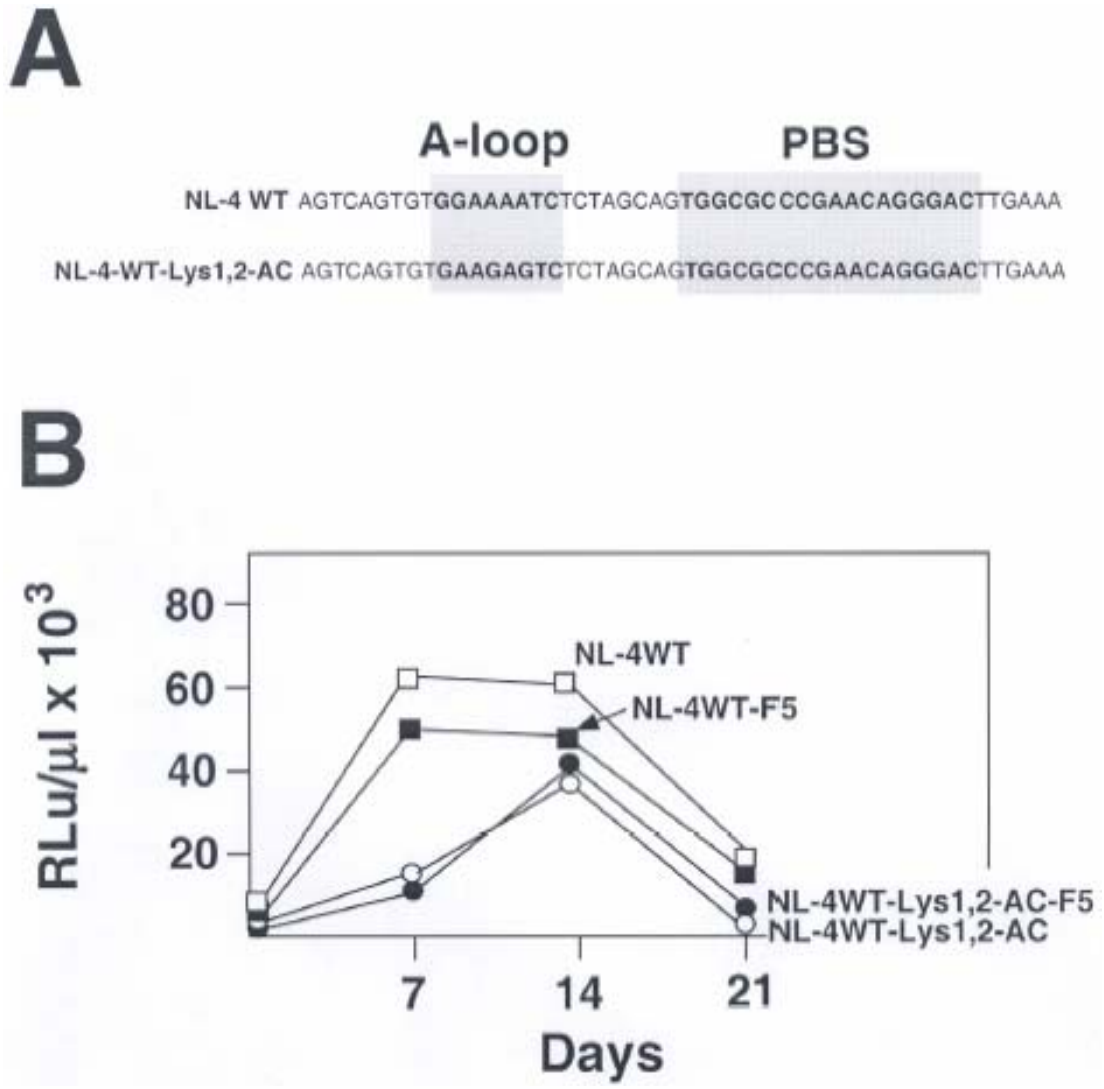

Figure 2. **HIV-1 Proviral genome with A-loop complementary to tRNA^{Lys1,2}.** HIV-1 genomes were constructed in which the A-loop region was modified to be complementary to tRNA^{Lys1,2} while retaining the PBS complementary to tRNA^{Lys,3} (shaded). These genomes, designated as NL4-WT-Lys 12-AC, were constructed to contain the wild type Gag-pol frameshift region or the frameshift region in which the lysine codons were altered to be specific for tRNA^{Lys1,2} (F5).

Panel B. Replication of wild type virus and HIV-1 with A-loop complementary to tRNA^{Lys1,2}. Viruses with the A-loop complementary to tRNA^{Lys1,2} and PBS complementary to tRNA^{Lys,3} were used to initiate infection in human PBMC. At Day 14, PHA activated human PBMC were added to the culture which were then allowed to incubate for a total of 28 days. Analysis of viral infection was done using supernatants and the JC53-BL assay. Data is representative of two independent experiments.

determined by the JC53-BL assay (Figure 3). Infections were established with equal amounts of infectious virus in PBMC. Analysis of the virus replication revealed that the mutation of the PBS to be complementary to $tRNA^{Met}$ severely compromised the production of infectious virus. Regardless of whether the Gag-pol frameshift was wild type or the codons had been mutated to be specific for $tRNA^{Lys1,2}$, the virus replicated at levels considerably less than that of the wild type virus. Analysis of the PBS for these viruses following replication revealed that they maintained complementarity with the 3' terminal nucleotides of $tRNA^{Met}$. Thus, though the A-loop and frameshift site were modified to optimize use of $tRNA^{Lys1,2}$, the virus still maintained a PBS complementary to $tRNA^{Met}$ following *in vitro* replication, demonstrating the intracellular availability of $tRNA^{Met}$ for primer selection was not influenced by the changes in the lysine codons.

Discussion

In the current study, we have further delineated crucial viral elements that are involved in selection of the tRNA primer used for reverse transcription. Modification of the HIV-1 genome in which the A-loop and PBS were altered to be complementary to $tRNA^{Lys1,2}$ forced HIV-1 to utilize this tRNA as the primer for replication. Consistent with previous studies, we found that alteration of the codons prior to the Gag-pol frameshift region enhanced the replication of the virus forced to use $tRNA^{Lys1,2}$. Analysis of the effects of the alteration of the A-loop or PBS alone revealed that complementarity of the tRNA with the PBS controls the preference for the tRNA primer to be selected. However, the A-loop modification is required for continued selection (as manifested by stability of PBS) of $tRNA^{Lys1,2}$ during *in vitro* culture.

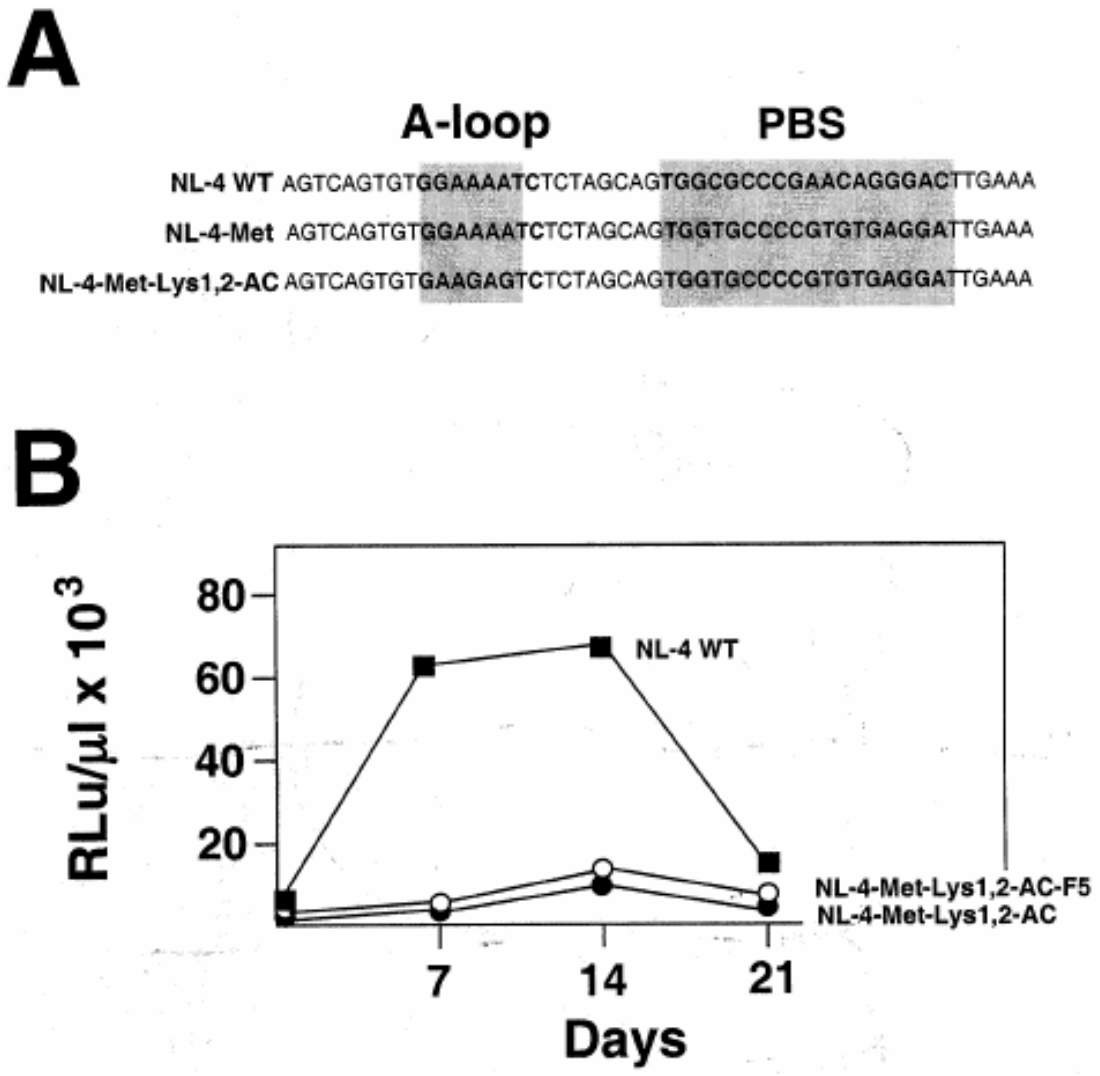

Figure 3. Characterization of HIV-1 with A-loop complementary to tRNA^{Lys1,2} and PBS complementary to tRNA^{Met}.

Panel A. HIV-1 proviruses were constructed in which the A-loop was modified to be complementary to tRNA^{Lys1,2} and the PBS modified to be complementary to tRNA^{Met} (shaded). These proviruses (NL4-Met-Lys12-AC) were then constructed in which the Gag-pol frameshift region was wild type or contained lysine codons specific for tRNA^{Lys1,2} (F5).

Panel B. Replication of viruses with A-loop complementary to tRNA^{Lys1,2} and PBS complementary to tRNA^{Met}. Replication of viruses was analyzed using human PBMC cultures. At Day 14, the cultures were re-fed with additional PHA stimulated PBMC. The supernatants at the designated days were analyzed for virus using the JC53-BL assay. For comparison, the growth of the wild type virus (NL4) is depicted, highlighting the low replication of viruses with the PBS complementary to tRNA^{Met}. Data is representative of two independent experiments.

Previous studies from this laboratory have described HIV-1 which have been forced to utilize $tRNA^{Lys1,2}$ as the primer (Kang and Morrow, 1999; Moore et al., 2004). In order to do this, mutations both in the A-loop region and PBS were needed to be complementary to the 3' terminal 18-nucleotides and anticodon of $tRNA^{Lys1,2}$. Since $tRNA^{Lys1,2}$ not only interacts with lysl-synthetase, but is also incorporated into HIV-1 virions, we anticipated that this virus would have replication characteristics similar to the wild type virus that uses $tRNA^{Lys,3}$. While this virus maintained a PBS complementary to $tRNA^{Lys1,2}$, it grew at levels that were lower than that of the wild type virus. Thus, even though $tRNA^{Lys1,2}$ can interact with the lysl-synthetase, it was not able to be selected and utilized efficiently by HIV-1 as the primer for reverse transcription, which supports that interaction with lysl-synthetase is not the sole requirement for primer selection. Previous studies from our laboratory have suggested a relationship between translation and primer selection (Kelly, Palmer, and Morrow, 2003; McCulley and Morrow, 2006). Studies using an *in vivo* complementation system have shown that tRNAs involved in translation were more efficiently selected as HIV-1 reverse transcription primers (McCulley and Morrow, 2006). Based on parallel studies of primer selection in MuLV and from our previous work on HIV-1, we found that codons prior to the Gag-pol frameshift (for HIV; the read through for MuLV) was enriched for codons that corresponded to tRNAs favored for primer selection (Palmer et al., 2006). Modification of the codons prior to the Gag-pol frameshift site so as to favor $tRNA^{Lys1,2}$ increased the infectivity of viruses that were forced to utilize $tRNA^{Lys1,2}$. Although, the level of replication of these viruses were not equal to that of the wild type virus, it was sufficiently improved to ask whether these viruses would still exhibit a preference for $tRNA^{Lys1,2}$ over $tRNA^{Lys,3}$. To address this

question, we analyzed the effects of mutating the A-loop region has a primer selection. As shown by our results, the $tRNA^{Lys1,2}$ PBS mutant with a wild type A-loop ($tRNA^{Lys,3}$) did not stably maintain the PBS complementary to $tRNA^{Lys1,2}$ even with the additional mutations in the Gag-pol frameshift region. Thus, even though the virus with the PBS complementary to $tRNA^{Lys1,2}$ had a replication advantage with the mutations in the Gag-pol frameshift region, the PBS was still unstable without the corresponding A-loop mutation. At first glance, this result would suggest that the A-loop mutation is dominant with respect to primer selection. However, mutation of the A-loop to be complementary to the anticodon of $tRNA^{Lys1,2}$, even with the additional mutations in the Gag-pol, did not force the virus to utilize $tRNA^{Lys1,2}$ when the PBS was complementary to $tRNA^{Lys,3}$. Furthermore, modification of the PBS to be complementary to $tRNA^{Met}$, which resulted in a virus with low infectivity, could not be forced to select $tRNA^{Lys1,2}$ with mutations in the A-loop and Gag-pol frameshift region. Collectively, the results point to a hierarchy between complementarity with the PBS, A-loop and codon use in the process of primer selection. The complementarity of the PBS with $tRNA^{Lys,3}$ and to some extent $tRNA^{Met}$, dictates the selection of these primers for reverse transcription. Complementarity of the A-loop with the anticodon of $tRNA^{Lys1,2}$ is important to stably utilize the tRNA for replication and the codon use near the Gag-pol frame shift region can enhance replication (i.e. selection) of virus designed to use $tRNA^{Lys1,2}$.

Previous studies from our laboratory have examined the effects that numerous A-loop-PBS modifications have on primer selection (Dupuy et al., 2003; Kang and Morrow, 1999; Kang, Zhang, and Morrow, 1997; Kang, Zhang, and Morrow, 1999; Ni and Morrow, 2006; Wakefield, Kang, and Morrow, 1996; Zhang et al., 1998). As a result of

these studies, we have developed a group of tRNAs that HIV-1 will use as primers for replication with the appropriate A-loop modifications. These tRNAs include $tRNA^{Lys1,2}$, $tRNA^{His}$, $tRNA^{Met}$, $tRNA^{Glu}$ and $tRNA^{Thr}$. Other tRNAs, including $tRNA^{Ile}$, $tRNA^{Ser}$ and $tRNA^{Tyr}$ were not selected and even with A-loop modifications the virus would revert to use $tRNA^{Lys,3}$. Based on our results that demonstrate complementarity with the PBS dictates tRNA selection, the intracellular availability of the tRNA primer is an important indicator of the stability of the PBS and replication capacity of the virus. Since some tRNAs are favored and others not, the results are consistent with the idea that a pool of tRNAs are available for primer selection. Undoubtedly, $tRNA^{Lys,3}$ is the most available tRNA within this pool since this tRNA is preferred for selection. Additional tRNAs within this pool could include $tRNA^{Lys1,2}$, $tRNA^{Met}$ as well as additional tRNAs that we have shown to be stably utilized by HIV-1 if additional mutations in the A-loop were made to be complementary to the anticodon of these tRNAs (e.g. $tRNA^{His}$) (Kang and Morrow, 1999; Kang, Zhang, and Morrow, 1999; Moore et al., 2004; Ni and Morrow, 2006). What determines the composition of tRNAs in the pool unclear. Based on our previous results and the result of this study, we speculate that the tRNAs involved in translation might establish a pool of tRNAs that could be accessed for primer selection. If this is the case, we would suspect that $tRNA^{Lys,3}$ is somehow enriched within this pool for selection. Alteration of the codon preference at a Gag-pol frameshift region had a drastic effect on the replication of viruses forced to use $tRNA^{Lys1,2}$ but did not affect the replication of viruses utilizing $tRNA^{Lys,3}$ (wild type) or $tRNA^{Met}$. It is possible that other regions of Gag might also be important to establish the tRNA pool that is enriched for

$tRNA^{Lys,3}$. It still remains to be determined what controls the composition of tRNAs for the primer selection pool.

Finally, additional considerations for the preferred selection of $tRNA^{Lys,3}$ should be considered. For example, unique structural features of $tRNA^{Lys,3}$ could facilitate the selection and use in reverse transcription (Benas et al., 2000). Indeed, studies have shown using *in vitro* and recently an *in vivo*, complementation system that unique features of the anticodon loop of $tRNA^{Lys,3}$ and in the selection and use in reverse transcription (McCulley and Morrow, 2006). Additional elements within the HIV-1 genome, such as the primer activation signal (PAS) and a region in U3 that acts to facilitate strand transfer during reverse transcription, could also play a role in the preferential use of $tRNA^{Lys,3}$ as the primer (Beerens, Groot, and Berkhout, 2001; Brule et al., 2000). Ultimately, the elucidation of the multiple viral elements in conjunction with a further delineation of the composition of the intracellular pool of tRNAs available for selection will provide further insights into the mechanism for the selection of the tRNA primer used in reverse transcription.

Materials and Methods

Plasmid construction

To construct the HIV-1 with altered codons near the Gag-pol frameshift region, pNL4-3 (Adachi et al., 1986) was partially digested with *Bcl*I. The 10,438 bp, provirus-containing fragment was gel-extracted and cloned into pUC19's *Bam*HI site. Several $tRNA^{Lys,3}$ codons upstream of the frameshift site were changed to $tRNA^{Lys1,2}$ codons by PCR amplification of the resulting plasmid using

5’GGATCCGGGCCCCTAGGAAGAAGGGCTGTTGGAAGTGTGGAAAGGAAGGA CACCAAATGAAGGATTG3’

and 5’-GAATTCATTTCTGTACAAATTTCTACTAATGC-3’ as primers. The amplicon was digested with *Apa*I and *Bcl*I and cloned into the parent plasmid to create pNL4-3(F5). The integrity of the inserted region was verified by sequencing.

To construct the HIV-1 clones, pUC119 HXB2 was used to mutate the PBS and/or the A-loop by following the manufacture’s instructions of the Qiuckchange II Site Directed mutagenesis Kit (Stratagene). We have previously described pUC11HXB2 with the PBS complimentary to human $tRNA^{Lys1,2}$ and $tRNA^{Met}$. The latter was used to create an A-loop with complementarity to the anticodon of $tRNA^{Lys1,2}$ with the following set of primers (5’ CCCTTTTAGTCAGTGTGAAGAGTC TCTAGCAGTGGTGCCCC 3’ and 5’ GGGGCACCACTGCTAGAGACTCTTCACACTGACTAAA AGGG 3’). To mutate the A-loop to be complimentary to the anticodon of $tRNA^{Lys1,2}$, we used pUC119 HXB2 along with the following primers (5’ CCCTTTTAGTCAGTGTGAAGAGTCTCTAG CAGTGGCGCCC 3’ and 5’ GGGCGCCACTGCTAGAGACTCTTCACACTGACTAAAAGGG 3’). *BssHII* and *HpaI* (New England Biolabs) were used to digest and release the HIV-1 fragment with the desired PBS and/or A-loop mutations. The mutated fragment was ligated into the pNL4-3 and pNL4-3(F5) backbone using T4 ligase (Promega) following the excision of the equivalent fragment in these plasmids with BssHII/SmaI and BssHII/SnaBI, respectively. The sequences of the newly synthesized plasmids were verified by sequencing.

Cell culture and DNA transfections

293T cells were transfected with plasmids containing different HIV proviral genomes using previously described methods (Moore et al., 2004). Transfections were done utilizing FuGENE transfection reagent according to the manufacturer's protocol. The transfections were incubated overnight at 37^{o}C and the medium was then replaced with fresh DMEM containing 10% FBS. After 48 hours all the supernatants were collected and stored at –80^{o}C until further analysis. The supernatant from transfected cells were assayed for HIV-1 p24 antigen and infectious virus using the JC53-BL assay (Moore et al., 2004).

Analysis of HIV-1 viral growth kinetics and infectivity

HIV-1 replication was determined using human peripheral blood mononuclear cells (PBMCs) as previously described. The PBMCs were stimulated using recombinant IL-2 and PHA, and were maintained as previously described. Infections were performed by inoculating 20 x 10^6 PHA stimulated PBMC with the volume of transfection supernatant containing the desired quantity of infectious virus as determined by the JC53-BL assay (Moore et al., 2004). The virus was allowed to adsorb for 2 hours at 37^{o}C in 5% CO_2. Virus/PBMC mixtures were transferred to 25 cm^2 flasks and the final volumes adjusted to 10 mL with RPMI-1640 containing 15% FBS and 30 units per mL of IL-2 as previously described (Moore et al., 2004). Infected PBMC cultures were maintained for 4 weeks by replacing half the volume of medium every three to five days without removing PBMC. Every 7 days, 1 mL of cell suspension was removed and centrifuged in an Eppendorf microfuge at 16,000 x g for 2 minutes. The supernatants were analyzed for

virus by HIV p24 antigen ELISA and JC53-BL assay (Derdeyn et al., 2000). Cell pellets were stored at –80^{o}C until processed for isolation of high-molecular weight (HWM) DNA for analysis of the PBS. Every 14 days an additional 5 x 10^{6} PHA-stimulated PBMCs were added to each culture.

Analysis of the proviral PBS region

Cell pellets from HIV-1 infected PBMC cultures were processed for isolation of HMW genomic DNA as previously described (Moore et al., 2004). Approximately 2 μg of each genomic DNA sample was PCR amplified using primers bracketing the U5-PBS region. Recombinant Taq polymerase was used for PCR amplification. Resulting PCR products were isolated and directly sequenced (Moore et al., 2004).

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DISCUSSION AND CONCLUSIONS

The process of reverse transcription is mediated by virally encoded RT and represents a crucial step in the retroviral life cycle [16-19]. RT necessitates a primer in order to initiate reverse transcription [83]. Although the primer of choice is not conserved among all retroviruses, HIV-1 RT constitutively utilizes cellular $tRNA^{Lys,3}$ [58]. Even though the mechanism of reverse transcription itself has been largely defined, it is still unclear how, when and why $tRNA^{Lys,3}$ is selected for HIV-1 reverse transcription. In pursuit of the answer, numerous mutational analyses of the viral genome have proved to be instrumental. The PBS represents the primary site of interaction between the vgRNA and the tRNA primer. HIV-1 can be forced to use many cellular tRNAs as primer for reverse transcription if the PBS complements the 3' 18 terminal nucleotides of a particular tRNA [145, 146, 148]. However, secondary vgRNA:tRNA interactions greatly influence primer usage [143, 144, 146, 154, 157, 158, 185-187]. The reports presented in this dissertation provide evidence supporting the coordination of primer selection, translation and vgRNA encapsidation.

Linking Primer Selection, Translation and Encapsidation

Previous studies have suggested a link between HIV-1 tRNA primer selection and encapsidation of vgRNA might be coupled with viral translation [137, 162]. In order to further investigate this relationship, a unique genetic approach was used in this study that

exploits utilization of initiator methionine tRNA ($tRNA^{Met(i)}$) and elongator tRNA methionine ($tRNA^{Met(e)}$) in eukaryotic translation. The two methionine tRNAs have the same anticodon sequence 5' CAU 3', and are charged with the same aminoacyl synthetase[182]. $tRNA^{Met(i)}$ interacts with eIFs and binds only to the P site of the small ribosomal subunit [181, 182]. It is used exclusively for initiation of translation. In contrast, $tRNA^{Met(e)}$ binds eEFs. During translational elongation, a methionine-charged $tRNA^{Met(e)}$ binds methionine codons in the ribosomal A site. Due to the functional differences, $tRNA^{Met(e)}$ and $tRNA^{Met(i)}$ are quite possibly located in separate cellular locales. Under the hypothesis that primer selection is coordinated with viral translation, forcing the virus to use $tRNA^{Met(i)}$ might impact virus replication.

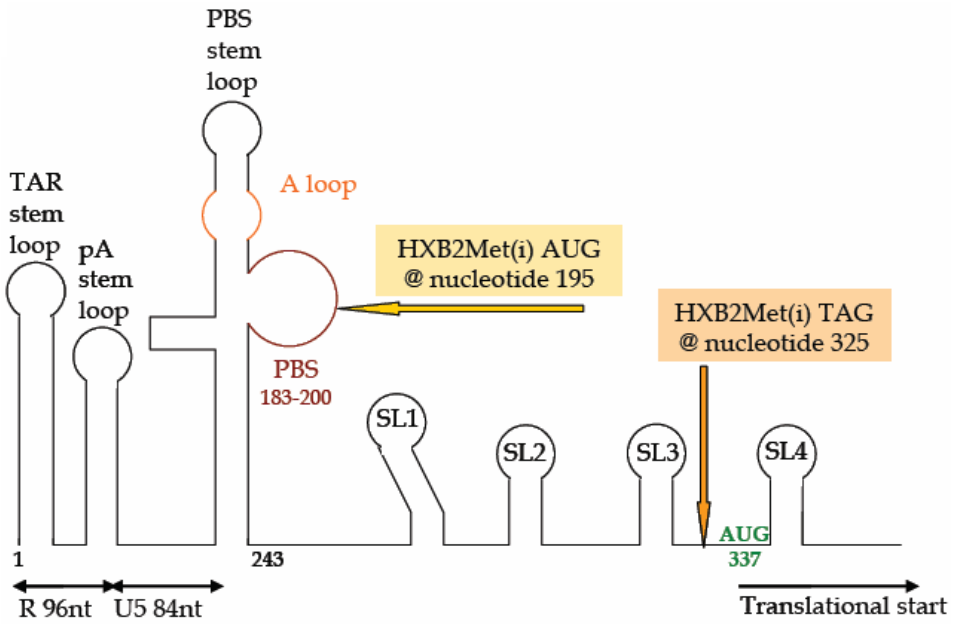
Previously, we have isolated, characterized and re-derived a replication competent HIV-1 mutant with PBS complementarity to the 18 3' terminal nucleotides of $tRNA^{Met(e)}$ (HXB2-Met(e)) [143, 157, 158]. For this study, we engineered a proviral clone with a PBS that was complimentary to the 3' 18 nucleotides of $tRNA^{Met(i)}$ (HXB2-Met(i)). Next we characterized the effects of the introduced mutations on viral viability. Following transfection of wild type and mutant proviral clones into 293 T cells, the virus containing supernatant was used to initiate an infection in SupT1 cells. Both HXB2-WT and HXB2-Met(e) produced a productive infection, measured by CAp24 antigen in culture supernatant. However, HXB2-Met(i) failed to infect SupT1 cells. To date, this was the only HIV-1 clone that uses a eukaryotic tRNA for reverse transcription unable to produce a productive infection. Therefore, we sought to further characterize HXB2-Met(i). We determined that both the producer (human embryonic kidney (293T)) cells and the target cells (SupT1) contained similar amounts of $tRNA^{Met(i)}$ and $tRNA^{Met(e)}$. Hence the relative

amounts of these tRNAs was not the limiting factor for primer selection. Using an endogenous reverse transcription reaction on transfected supernatant, we demonstrated that radionucleotide incorporation levels in negative strong stop DNA synthesis were equivalent between HXB2-Met(e) and HXB2-Met(i). Hence, $tRNA^{Met(i)}$ could be selected and used for priming reverse transcription We next turned to examining HXB2-Met(i) itself. The JC53-BL β-galactosidase assay was utilized to determine production of infectious viral particles in transfected supernatant. HXB2-Met(e) transfection resulted in production of virus at approximately 20% of wild type level. The decrease of infectious virus production was expected, since similar findings have been shown for other HIV-1 PBS mutants [188, 189]. However, HXB2-Met(i) had severely reduced infectious virus production at approximately 2% of wild type. In order to determine the cause of the defect, we analyzed culture supernatants for CAp24 antigen levels with an ELISA. This assay effectively measures the amount of all particles produced from transfection, whether infectious or not. Previous studies in the lab demonstrated PBS mutations reduce infectious particle production, albeit the total amount of CAp24 antigen remains unaltered [145, 157, 159]. Over a range of equivalent plasmid transfection amounts, the amount of HXB2-WT and HXB2-Met(e) CAp24 antigen produced was virtually identical compared to an approximately 50% reduction in HXB2-Met(i) production. Thus, HXB2-Met(i) was not only poorly infectious, but its transfection yielded less virus. One explanation for this phenomenon was that selection of $tRNA^{Met(i)}$ as a primer for reverse transcription was occurring at an inappropriate time during translation, causing the viral genomic RNA to be packaged sooner. In turn, this would disrupt or at least alter the amount of $Pr160^{Gag\text{-}pol}$ synthesized. Since virus maturation and

viability depends on virally encoded PR, RT, and IN synthesized from the Pol part of $Pr160^{Gag\text{-}pol}$, we hypothesized that proteolytic processing in HXB2-Met(i) was altered. Since CAp24 ELISA poorly recognizes unprocessed $Pr55^{Gag}$, we tested the theory via western blot. The polyclonal antibodies used in this assay recognize both $Pr55^{Gag}$ and CAp24. HXB2-WT, HXB2-Met(e), and HXB2-Met(i) all had greater amounts of CAp24 than $Pr55^{Gag}$, indicating that proteolytic processing was not compromised by introducing an alternate PBS. The amount of $Pr55^{Gag}$ in HXB2-Met(i) is 10 fold lower than in both HXB2-WT and HXB2-Met(e). Additionally, it is interesting that the CAp24/$Pr55^{Gag}$ ratio in HXB2-WT and HXB2-Met(e) is approximately 3 to 5, whereas it is 10 to 50 in HXB2-Met(i). Although there is no apparent explanation for the differences of CAp24/$Pr55^{Gag}$, it is clear that the data indicate that proteolytic processing of $Pr160^{Gag\text{-}pol}$ was possible.

The canonical HXB2 *gag* start codon is located at nt 337, some 150 nucleotides downstream of the PBS [39](Figure 8). In reexamining the $tRNA^{Met(i)}$ 3' 18 terminal nucleotide sequence, we noticed that it contains an AUG. Most probably, the major reason for the defects observed in HXB2-Met(i) stems from the inadvertent introduction of an AUG codon in the PBS. Hence, if translation initiated from this misplaced start codon, $Pr55^{Gag}$ and $Pr160^{Gag\text{-}pol}$ production would be compromised. Therefore, we constructed a HXB2-Met(i) with an A to G mutation (HXB2-Met(i)AG) at position 13 of the PBS. This point mutation changed the AUG to GUG and ablated its function. Characterization of HXB2-Met(i)AG showed that virus production was restored following transfection. Infectivity levels increased and were now similar to those observed with HXB2-Met(e). Unlike HXB2-Met(i), HXB2-Met(i)AG was able to

A



B

Provirus	PBS Sequence
HXB2 WT	5' TGG CGC CCG AAC AGG GAC 3'
HXB2 Met(e)	5' TGG TGC CCC GTG TGA GGA 3'
HXB2 Met(i)	5' TGG TAG CAG AGG ATG GTT 3'
HXB2 Met(i) AG	5' TGG TAG CAG AGG GTG GTT 3'

Figure 8. Schematic representation of the HXB2-Met(i) 5' UTR.
Panel A. By introducing a PBS complementary to $tRNA^{Met(i)}$, the new viral construct contains an AUG start codon in the primer binding site (PBS). The canonical start codon is located at nt 337, while the PBS stretches from nt183 to 200.
Panel B. PBS sequences of various HIV-1 clones. Note the A to G mutation in HXB2-Met(i)AG. This mutation was introduced to abrogate the AUG in HXB2-Met(i).

produce a productive infection in SupT1 cells. Moreover, our endogenous reverse transcription assay data were corroborated. Indeed, nothing precluded usage of $tRNA^{Met(i)}$ in reverse transcription, demonstrated by PBS sequence analysis of integrated HXB2-Met(i)AG provirus.

Unexpected results were observed in analyzing the PBS sequence of integrated proviral DNA following extended replication of HXB2-Met(i)AG in SupT1 cells. The dsDNA product resulting from reverse transcription should have one strand with the PBS retaining the A to G mutation (stemming from the packaged vgRNA), while the other strand should contain a PBS complimentary to $tRNA^{Met(i)}$ (acquired from $tRNA^{Met(i)}$ during plus strand synthesis) (Figure 9). Following integration, the provirus would contain a "bubble" at position 13 of the PBS. Unless a bias occurs during repair, half of the infected cells would have a PBS complementary to $tRNA^{Met(i)}$, while the other half would keep the $tRNA^{Met(i)}$ PBS with the A to G point mutation. If this were the case, with every replication cycle there would be less and less integrated proviruses with the A to G point mutation. Since HXB2-Met(i) is noninfectious due to the AUG codon in the PBS, eventually we would not be able to recover infectious virus. Surprisingly, we found that more virus was produced in the culture as time progressed. Moreover, the PBS sequence of the integrated provirus retained the A to G point mutation, suggesting preferential selection and packaging of vgRNA containing this mutation.

Collectively, presented data suggest that there is a link between primer selection, viral translation, and packaging of vgRNA. Multi component complexes consisting of aminoacyl synthetases facilitate efficient translation by forming local microenvironments of tRNA and host proteins [190-192]. Possibly, HIV-1 has evolved mechanisms to

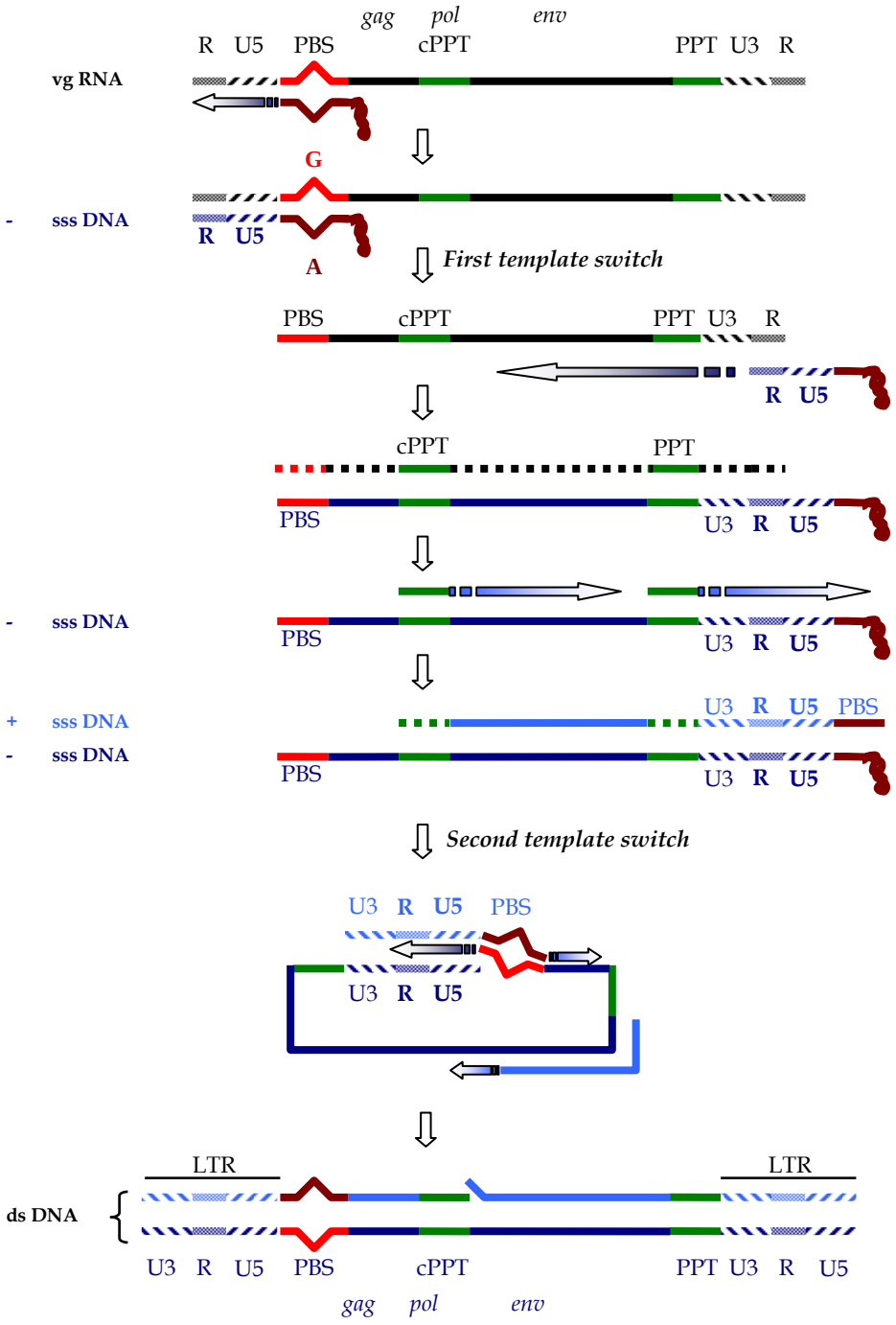


Figure 9. Reverse transcription of HXB2-Met(i) AG
Viral genomic RNA (vgRNA) primer binding site sequence (PBS) (red) contains a G at position 13. A "bubble" forms between the $tRNA^{Met(i)}$ (dark red) that binds to the PBS, since the $tRNA^{Met(i)}$ contains an A at the same position. Following completion of reverse transcription, the dsDNA will contain a mismatch at position 13 of the PBS, since the PBS sequences are derived from vgRNA (during –sssDNA synthesis) and $tRNA^{Met(i)}$ (during +sssDNA synthesis). Following integration, cellular enzymes will correct the mismatch in the provirus.

acquire a tRNA from these locales of increased tRNA concentration during translation. $Pr160^{Gag\text{-}pol}$ has been implicated in primer selection. Consistent with observations in wild type HIV-1 virions, pseudovirions composed of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}pol}$ exhibited enrichment of $tRNA^{Lys,3}$, unlike pseudovirions composed of only $Pr55^{Gag}$ [139, 193, 194]. Other studies have linked $Pr55^{Gag}$ with encapsidation through interaction of the NC domain of the polyprotein with vgRNA Ψ [71, 137, 195, 196]. Our data support the idea that translated vgRNA is packaged into virions, since HXB2-Met(i)AG is amplified in culture even though $tRNA^{Met(i)}$ is selected as the primer for reverse transcription. Nonetheless additional studies will be necessary to more specifically define the mechanism linking translation with primer selection and encapsidation.

Selection Preference for the HIV-1 Primer tRNA Used in Reverse Transcription is Controlled by Intracellular Availability and the Interaction with the U5-PBS

It has long been shown that mutating the HIV-1 PBS to be complementary to a tRNA other than $tRNA^{Lys3}$, forces the virus to select and use that particular tRNA as the primer for reverse transcription for at least a limited time. In some cases, additional mutations in the A-loop extended the time some of these alternate tRNAs were used before reverting to utilizing the preferred $tRNA^{Lys3}$ [143, 144, 154, 158, 159, 185, 197]. tRNAs are integral components of the cellular translation machinery. Therefore, it stood to reason to postulate that protein biosynthesis and the process of HIV-1 primer selection are connected. Indeed, evidence has been put forth that suggested a relationship between primer selection and translation [161]. Moreover, a region located upstream the HIV-1 *gag-pol* frameshift site has linked primer selection with vgRNA translation by

implicating codon usage with alternate tRNA usage stability [162]. Therefore, three regions (PBS, A-loop and the *gag-pol* frameshift site) have been identified and implicated in HIV-1 tRNA primer selection which seems to occur at the level of vgRNA translation. In order to build upon our studies, we have further characterized the relative involvement of these crucial viral elements in primer selection.

Altering the PBS to be complementary to the 3' 18 nucleotides of $tRNA^{Lys1,2}$ forces the virus to use this tRNA for priming reverse transcription [156]. However, after extended culturing, the virus reverted to using $tRNA^{Lys3}$ as the primer. Additional mutations conferring complementarity of the A-loop to the anticodon of $tRNA^{Lys1,2}$ were sufficient to force HIV-1 to continually select and use $tRNA^{Lys1,2}$ in reverse transcription [159, 188]. Since $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ not only interact with the lysyl synthetase but are also incorporated into virions, we expected replication kinetics of the mutant virus to be similar to wild type [139, 163]. However, the mutant virus exhibited delayed replication kinetics and grew at lower levels in comparison to wild type, indicating that the interaction with lysyl synthetase is not the sole requirement for primer selection. A connection between primer selection and translation has been suggested based on complementation with $ytRNA^{Phe}$ and *Ec*$tRNA^{Lys3}$ [160, 161, 198, 199]. Specifically these studies showed that tRNAs are more efficiently selected as HIV-1 reverse transcription primers if they enters the biosynthetic pathway. Examination of the sequence upstream of the *gag-pol* frameshift site, revealed that it was enriched for codons which correspond to tRNAs favored for primer selection [162]. This discovery suggested that these codons might influence tRNA availability for acquisition and use in reverse transcription. To test this possibility all lysine codons were mutated so that they would

interact with the $tRNA^{Lys1,2}$ anticodon. The new HIV-1 clone was named the frameshift mutant (F5). The previously characterized HIV-1 with PBS and A-loop complementarity to $tRNA^{Lys1,2}$, exhibits poor replication kinetics in culture. In contrast, if these mutations are placed in the F5 background, virus replication is strongly enhanced. Although improved, replication levels did not reach those of wild type HIV-1. However, the increase was sufficient enough to merit determining the influence of the frameshift mutations in preferential selection of $tRNA^{Lys1,2}$ over $tRNA^{Lys3}$. In order to further characterize the influence of the PBS, A-loop and the frameshift site, we constructed HIV-1 proviral clones so that the three regions would have various combinations of tRNA complementarity. Next, the effects of these mutations on primer selection were analyzed.

Our approach consisted of comparing previously described HIV-1 clones with newly engineered HIV-1 proviruses. All proviral clones were transfected into 293T cells and the viral supernatant was used to initiate productive infection in peripheral blood mononuclear cells (PBMCs). At various time points, the culture supernatant was assayed for production of infectious virus and a portion of the cells was isolated in order to determine the sequence of the PBS in integrated proviruses.

Three experimental sets were designed based on PBS complementarity of the viruses. In the first set we compared viruses in which the PBS was complementary to $tRNA^{Lys1,2}$ but exhibited variations of the A-loop and frameshift regions (Table 3). NL-4Lys1,2-F5 is the newly constructed proviral clone. Consistent with previous findings, NL-4Lys1,2-AC replicated poorly, albeit it retained the PBS complementarity to

$tRNA^{Lys1,2}$. In contrast, NL-4Lys1,2-AC-F5 replication was enhanced. This virus replicated almost as well as wild type and reversion to $tRNA^{Lys3}$ was not observed. In the

Table 3. Viruses used in experimental set 1.

Virus	PBS	A-loop.	Frameshift	PBS reversion to $tRNA^{Lys3}$
NL-4WT	$tRNA^{Lys3}$	$tRNA^{Lys3}$	WT	N/A
NL-4Lys1,2	$tRNA^{Lys1,2}$	$tRNA^{Lys3}$	WT	Yes
NL-4Lys1,2-AC	$tRNA^{Lys1,2}$	$tRNA^{Lys1,2}$	WT	No
NL-4Lys1,2-F5	$tRNA^{Lys1,2}$	$tRNA^{Lys3}$	F5	Yes
NL-4Lys1,2-AC-F5	$tRNA^{Lys1,2}$	$tRNA^{Lys1,2}$	F5	No

NL-4Lys1,2 and NL-4Lys1,2-F5 infected cultures, a delay of replication was seen in addition to a reversion of the viruses to use $tRNA^{Lys3}$ as the primer for reverse transcription (marked by an increase of viral replication at day 14). Based on these observations, it would seem that the A-loop sequence is dominant with respect to primer selection. However, results from the other two pairs of mutants did not support this idea.

In the second experimental set, the PBS of the viruses is complementary to $tRNA^{Lys3}$ (wild type). Constructed clones have different combinations of A-loop and frameshift region complementarity and interact with either $tRNA^{Lys3}$ or $tRNA^{Lys1,2}$ (Table 4). The newly constructed viruses are NL-4WT-F5, NL-4WT-Lys1,2-AC and NL-4WT-

Table 4. Viruses used in experimental set 2.

Virus	PBS	A-loop	Frameshift	PBS reversion to $tRNA^{Lys1,2}$
NL-4WT	$tRNA^{Lys3}$	$tRNA^{Lys3}$	WT	N/A
NL-4WT-F5	$tRNA^{Lys3}$	$tRNA^{Lys3}$	F5	No
NL-4WT-Lys1,2-AC	$tRNA^{Lys3}$	$tRNA^{Lys1,2}$	WT	No
NL-4WT-Lys1,2-AC-F5	$tRNA^{Lys3}$	$tRNA^{Lys1,2}$	F5	No

Lys1,2-AC. NL-4WT-F5 had a slight delay in replication and virus production. The A-loop alteration in NL-4WT-Lys1,2-AC resulted in a delay in replication and an overall lower peak of virus production in comparison to wild type. Similar results were observed in the NL-4-Lys1,2-AC-F5 infected culture. None of the tested viruses could be forced to utilize $tRNA^{Lys1,2}$ as the primer for reverse selection. Moreover, even though NL-4WT-Lys1,2-AC-F5 has two of the three viral RNA regions (A-loop and frameshift) interact with $tRNA^{Lys1,2}$, these alterations were insufficient for the $tRNA^{Lys3}$ PBS to become complementary to $tRNA^{Lys1,2}$. These data indicate that the PBS is the major determinant in HIV-1 primer selection.

In the third set of experiments, viruses with PBS complementarity to $tRNA^{Met}$ were examined (Table 5). As it was the case with previous experiments, tested viral

Table 5. Viruses used in experimental set 3.

Virus	PBS	A-loop	Frameshift	PBS reversion to $tRNA^{Lys3}$ or $tRNA^{Lys1,2}$
NL-4WT	$tRNA^{Lys3}$	$tRNA^{Lys3}$	WT	N/A
NL-4Met-Lys1,2-AC	$tRNA^{Met}$	$tRNA^{Lys1,2}$	WT	No
NL-4Met-Lys1,2-AC-F5	$tRNA^{Met}$	$tRNA^{Lys1,2}$	F5	No

clones differ in their A-loop and frameshift region sequences. NL-4Met-Lys1,2-AC and NL-4Met-Lys1,2-AC-F5 are newly constructed and contain an A-loop complementary to $tRNA^{Lys1,2}$ in a either WT or F5 background, respectively. The previously characterized HIV-1 clone with a PBS complementary to $tRNA^{Met}$ (NL-4Met), was shown to first convert to using $tRNA^{Lys1,2}$ for reverse transcription (under certain circumstances) before finally reverting to utilize $tRNA^{Lys3}$ [157, 159]. Therefore by constructing the last pair of

clones we attempted to facilitate this conversion. Nonetheless, replication for both NL4Met-Lys1,2-AC and NL4Met-Lys1,2-AC-F5 was severely compromised. Additionally, the PBS sequences of integrated provirus remained complementary to $tRNA^{Met}$, indicating that we were unable to force the usage of $tRNA^{Lys1,2}$ for reverse transcription. Therefore, as was the case with NL-4 mutants with PBS complementarity to $tRNA^{Lys3}$, the A-loop and the frameshift region (either alone or in combination) are not sufficient for PBS reversion to $tRNA^{Lys1,2}$.

Collectively, these data indicate that there is a hierarchical influence of the PBS and A-loop sequences and codon utilization in the gag-pol frameshift site in primer selection. PBS complementarity to $tRNA^{Lys3}$ and $tRNA^{Met}$ causes selection of these tRNAs for priming reverse transcription. In viruses with PBS complementarity to $tRNA^{Lys1,2}$, the A-loop complementarity to $tRNA^{Lys1,2}$ stabilizes the use of this tRNA in replication while the codon usage in the gag-pol frameshift site enhances its selection.

From extensively characterized A-loop-PBS HIV-1 mutants, we have identified a group of cellular tRNAs that are used as primers in reverse transcription. These tRNAs include $tRNA^{Lys1,2}$, $tRNA^{His}$, $tRNA^{Met(e)}$, $tRNA^{Glu}$, and $tRNA^{Thr}$. On the other hand, $tRNA^{Ile}$, $tRNA^{Ser}$, and $tRNA^{Tyr}$ could not be continuously selected even with appropriate A-loop modifications. Based on our results which indicate that PBS complementarity is responsible for tRNA selection, tRNA availability in the intracellular milieu is an important factor that influences PBS stability and replication of the virus. Only certain tRNAs are conducive for selection, which is in line with the idea that a pool of tRNAs are available for acquisition. Since $tRNA^{Lys3}$ is the HIV-1's cognate primer, it would seem likely that this tRNA is most available. However, HIV-1 can be forced to continuously

select $tRNA^{Lys1,2}$ and $tRNA^{Met}$, indicating that these tRNAs are also found within this pool.

Overall, the results of this study show that while the PBS is the major determinant of tRNA primer utilization for reverse transcription, the A-loop influences continuous usage of a particular tRNA. Moreover, it seems that a pool of tRNAs eligible for use in reverse transcription is established, albeit it is still what determines its composition. Therefore it will be the focus of future studies.

It is clear that $tRNA^{Lys3}$ is the primer of choice for HIV-1. In addition to the PBS, A-loop and the frameshift, other regions of viral RNA may be involved in preferential selection of $tRNA^{Lys3}$. These include the primer activation site (PAS) and a region within the U3 which facilitates strand transfer during reverse transcription [187, 200]. Moreover, sequence and structural features unique to $tRNA^{Lys3}$ might impact its preferential selection.

FUTURE DIRECTIONS

tRNA primer selection represents a crucial step in HIV-1 replication. Although many studies over the past 15 years have significantly advanced out understanding of primer selection, the exact mechanism still remains to be resolved. The reports presented in this dissertation have characterized the role of previously identified regions of the viral genomic RNA in primer selection. Moreover, a genetic model has linked primer selection, translation of the viral genome and encapsidation. Still, future studies are warranted to link all the elements into a concrete mechanism.

We provide evidence that the codon usage upstream of the *gag-pol* frameshift site influences primer selection by mutating 3 codons that interact with $tRNA^{Lys3}$ to bind the $tRNA^{Lys1,2}$ anticodon. However, the relative contributions of these codons to the enhanced replication of HIV with both PBS and A-loop complementarity to $tRNA^{Lys1,2}$ remains unknown. Mutational studies will provide insight whether particular codon(s) has the most prominent influence. Furthermore, it would be interesting to determine whether replication of wild type HIV-1 can be elevated by changing the 2 codons that interact with $tRNA^{Lys1,2}$ to bind $tRNA^{Lys3}$. If replication is enhanced, these results would further establish the importance of the frameshift region in primer selection. Another possibility requires engineering HIV-1 PBS mutant complementary to *Ec*$tRNA^{Lys3}$ with equivalent combinations of PBS, A-loop and frameshift mutations, and determining the effects on primer selection. This is a viable option, since it has been recently shown that *Ec*$tRNA^{Lys3}$ can be used as primer for reverse transcription provided that the PBS is

mutated to interact with its 3' terminal 18 nucleotides [199]. It is important to note that none of these mutations alter the amino acid sequence of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}pol}$. Therefore, the observed results from neither the performed studies nor from proposed experiments, influence potential roles of these proteins in primer selection.

tRNAs are predominantly complexed with either their cognate aminoacyl synthetase or protein factors involved in translation. The most plausible explanation of tRNA primer selection by HIV-1 occurs at a point when the tRNA is in an unbound state. It is as of yet undetermined at what point during tRNA turnover HIV-1 is able to capture a free tRNA. During ribosome pausing at the frameshift site, an increased concentration of transiently free tRNAs exiting the ribosome could be captured. Another possibility is that the tRNAs are acquired as they are released from the stalled ribosome. tRNA secondary structures can be modified to induce or relieve pausing. Therefore, future experiments could potentially delineate how tRNAs are captured.

LysRS has been shown to be selectively packaged into the HIV-1 virion [163, 164, 201]. Moreover, previous studies have shown that eEF1A is incorporated into the virion, suggesting translation elongation proteins are also involved in primer selection [202, 203]. The translation initiation complex consists of eIF2-GTP-$tRNA^{Met(i)}$ [169]. Determining whether virions are enriched for eIF2 in our HXB2-Met(i)AG system will help further elucidate primer selection by identifying the putative proteins involved in the process.

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