Elements in a tRNA primer required for the infectivity of human immunodeficiency virus Type 1.

Qin Yu
University of Alabama at Birmingham

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ELEMENTS IN A tRNA PRIMER REQUIRED FOR THE INFECTIVITY OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

by

QIN YU

A DISSERTATION

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

BIRMINGHAM, ALABAMA

2000
Human immunodeficiency virus type 1 (HIV-1) uses tRNA\textsubscript{Lys} as a primer for reverse transcription. The 3'-end nucleotides of tRNA\textsubscript{Lys} bind to the primer binding site (PBS) of HIV-1 RNA genome for initiation of reverse transcription. HIV-1 can use many other tRNAs as primers in vivo when its PBS was mutated to be complementary to these tRNAs. Although RNA or DNA oligonucleotides complementary to the PBS of HIV-1 can serve as reverse transcription primers in vitro, they are not effective primers for HIV-1 in vivo. The research in this dissertation investigated elements in a tRNA that are required for the primer selection and use in HIV-1.

A complementation system was established in which a mutant HIV-1 relies on exogenous yeast tRNA\textsubscript{Phe} as the reverse transcription primer. The mutant HIV-1, designated as psHIV-Phe, contains a PBS complementary to yeast tRNA\textsubscript{Phe}. The infectivity of psHIV-Phe depended on the availability of yeast tRNA\textsubscript{Phe}. To identify elements of tRNA\textsubscript{Phe} important for primer selection and use in psHIV-Phe, a series of tRNA\textsubscript{Phe} mutants were generated and tested for their capacity to rescue psHIV-Phe. The results demonstrated that the anticodon and T\textsubscript{Y}C stem-loops of tRNA\textsubscript{Phe} are important for psHIV-Phe infectivity, whereas the D stem-loop is not required for rescuing the virus. Further analyses indicated that the base pairs in the acceptor stem and the nucleotide
sequence of the TΨC loop of tRNA$^{\text{Phe}}$ were critical for the virus rescue. Taken together, these results have revealed structural and sequence requirements in the tRNA for the primer selection and use in HIV-1 reverse transcription.
ACKNOWLEDGMENTS

I first thank my mentor, Dr. Casey Morrow, for his support and guidance throughout the years. I have learned a lot from him about how to design and conduct scientific research. I am grateful for his kindness and patience.

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Also, I acknowledge my parents, Faxin and Suqun, for their love and all the hardship they went through to support me. Also, a word of gratitude goes to my brothers, Jin and Xin, and their families. They have been very supportive and encouraging.

I especially thank my husband, Xiaoyong, for his undying support and love. Without him I would not have made it this far.
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### INTRODUCTION

1. Examples of the tRNAs used by retroviruses and retrotransposons as reverse transcription primers...

2. Complementarity between 3' terminal nucleotides of tRNA and primer binding site is a major determinant for selection of the tRNA primer used for initiation of HIV-1 reverse transcription.

1. HIV-1 with alternative PBS predominantly used the cognate tRNA as primer in initial round of replication...

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**COMPLEMENTARITY BETWEEN 3' TERMINAL NUCLEOTIDES OF tRNA AND PRIMER BINDING SITE IS A MAJOR DETERMINANT FOR SELECTION OF THE tRNA PRIMER USED FOR INITIATION OF HIV-1 REVERSE TRANSCRIPTION**

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ELEMENTS OF THE tRNA ACCEPTOR STEM AND \(\Psi\)C STEM-LOOP REQUIRED FOR HIV-1 INFECTION

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<td>A</td>
<td>nucleotide containing an adenine base</td>
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<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>C</td>
<td>nucleotide containing a cytosine base</td>
</tr>
<tr>
<td>CA</td>
<td>capsid protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cPPT</td>
<td>central polypurine tract</td>
</tr>
<tr>
<td>CTS</td>
<td>central terminal sequence</td>
</tr>
<tr>
<td>D</td>
<td>dihydrouridine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EF</td>
<td>elongation factor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>env</td>
<td>retrovirus envelope protein gene</td>
</tr>
<tr>
<td>Env</td>
<td>retrovirus envelope protein</td>
</tr>
<tr>
<td>G</td>
<td>nucleotide containing a guanine base</td>
</tr>
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<td>gag</td>
<td>retrovirus structural protein gene</td>
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<td>Gag</td>
<td>retrovirus gag-encoded precursor polyprotein</td>
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<td>Abbreviation</td>
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<tr>
<td>Gag-Pol</td>
<td>retrovirus gag and pol encoded precursor polyprotein</td>
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<td>GLS</td>
<td>the gag leader sequence</td>
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<tr>
<td>gp</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>gpt</td>
<td>xanthine-guanosine phosphoribosyl transferase</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HTLV</td>
<td>human T-cell leukemia virus</td>
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<tr>
<td>IN</td>
<td>integrase</td>
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<tr>
<td>LTR</td>
<td>long terminal repeat</td>
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<tr>
<td>MA</td>
<td>matrix protein</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MLV</td>
<td>murine leukemia virus</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>NC</td>
<td>nucleocapsid protein</td>
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<tr>
<td>nt</td>
<td>nucleotide(s)</td>
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<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
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<td>NTP</td>
<td>nucleotide triphosphate</td>
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<td>PBS</td>
<td>primer binding site</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PIC</td>
<td>preintegration complex</td>
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<tr>
<td>PR</td>
<td>protease</td>
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<tr>
<td>PPT</td>
<td>polypurine tract</td>
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R  the repeat region of retrovirus genome
RNA ribonucleic acid
RNase ribonuclease
RRE the Rev response element
RT reverse transcriptase
SIV simian immunodeficiency virus
SU surface domain of Env
SV40 simian virus 40
T nucleotide containing a thymine base
TAR trans-activating response elements in HIV RNA
TCA trichloroacetic acid
TM transmembrane domain of Env
tRNA transfer RNA
U nucleotide containing a uracil base
UTP uridine triphosphate
U3 the 3' unique region of a retrovirus genome
U5 the 5' unique region of a retrovirus genome
VLP virus-like particle
VSV-G vesicular stomatitis virus G glycoprotein
-sssDNA minus-strand strong-stop DNA
Ψ pseudouridine
INTRODUCTION

Overview

Human immunodeficiency virus (HIV) is a member of the large and diverse RNA virus family called retroviridae, also known as retroviruses. The hallmark of retroviruses is the reverse transcription of the viral RNA into a DNA duplex and the subsequent integration of this DNA into the chromosome of the cell. Retroviruses also share other common features. Retrovirus virions are usually 80-110 nm in diameter and have a lipid envelope containing viral glycoproteins. The 7-12 kb retroviral genomic RNA is a linear positive-sense RNA containing three major open reading frames: gag, pol, and env. Retroviruses are further divided into seven groups (genera) based on evolutionary relatedness (125). Three groups — the lentivirus genus, the spumavirus genus, and the human T-cell leukemia virus/bovine leukemia virus genus — are classified as complex retroviruses because of the presence of additional open reading frames coding “accessory” proteins in their genomes. Lentiviruses and spumaviruses are the only two groups of retroviruses that are not oncogenic. Lentiviruses cause disease by inducing loss of function of specific cells, whereas spumaviruses cause no known disease.

Lentiviruses have been isolated from a few animal species. HIV is the only known human lentivirus. HIV can be divided into two subtypes, HIV type 1 (HIV-1) and type 2 (HIV-2). HIV-1 is widely spread throughout the world and is the major cause of AIDS, whereas HIV-2 is primarily isolated in West Africa and is less pathogenic. HIV-1 has been the subject of intense investigation for the past two decades. HIV is transmitted

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by direct sexual contact, by blood or blood products, and from mother to infant. HIV-1 infection causes a decline of the CD4+ T lymphocytes, which eventually results in opportunistic infections and neoplasms. According to the report on the global HIV/AIDS epidemic from World Health Organization in 1999, approximately 34 million people were living with HIV/AIDS at the end of 1999. With 2.8 million AIDS deaths in 1999, HIV/AIDS has now become the fourth leading cause of mortality, and its impact is continuing to increase. Molecular studies of HIV-1 replication have led to the development of a number of anti-HIV-1 drugs. However, the high mutation rate and persistent (latent) infection of HIV-1 have hampered the development of vaccines and more successful therapeutics. Better understanding of the molecular biology of HIV-1 is needed to achieve effective prevention and management of HIV-1 infection.

The research presented in this dissertation focuses on the mechanism by which the tRNA primer is selected and used for HIV-1 reverse transcription. As a general introduction, the following sections review the molecular biology of HIV-1 replication and the general properties of tRNA.

HIV-1 Virion and Viral Components

This section starts with a brief description of the HIV-1 virion, followed by information on each component of the virus.

Virion

A mature HIV-1 virion is a spherical particle with a diameter of around 110 nm. It consists of 12 viral proteins, 1 genomic RNA, host cell-derived proteins, and tRNAs
The viral capsid protein forms the shell structure of the nucleoprotein core (Fig. 1). Inside this core, there are two copies of genomic RNA, eight viral proteins (nucleocapsid [NC], reverse transcriptase [RT], integrase [IN], protease [PR], p6, Vif, Vpr, and Nef), and a variety of host tRNAs. Among the encapsulated tRNAs, tRNA^{Lys}_{1,2,3} are the dominant species. The nucleoprotein core is surrounded by the viral matrix protein and a lipid bilayer membrane. The viral envelope protein is anchored in this membrane through its transmembrane domain (TM or gp41), with its surface domain (SU or gp120) projecting from the outer surface of the membrane. Host cell-derived proteins—including cyclophilin A, major histocompatibility complex (MHC), β-microglobulin, and ubiquitin—are also present in the membrane (5).

**Genomic RNA**

The genome of HIV-1 is a positive-sense RNA, about 9.5 kb in length. A dimer of two genomic RNA molecules is incorporated into the virion, which is responsible for the high recombination rate in HIV reverse transcription. HIV-1 genomic RNA encodes nine open reading frames (Fig. 2). Three of the open reading frames, gag, pol, and env, occupy the body of the viral genome. Six open reading frames encoding accessory proteins are located between pol and env or downstream from env. Besides these open reading frames, additional noncoding sequences are present at both ends of the viral RNA (Fig. 2). The 5’ end of the genomic RNA contains a repeat sequence (R) and a sequence unique to the 5’ end (U5). The 3’ end contains the same repeat sequence (R) and a sequence unique to the 3’ end (U3). These RNA sequences along with other important
FIG. 1. Schematic cross section through an HIV-1 virion. Major components of the virion are illustrated.
FIG. 2. Genetic organization of HIV-1 RNA genome. The open boxes represent the relative positions of the nine open-reading frames. Major RNA elements in the genome are shown in the approximate locations.
RNA elements in the viral genome are described as follows (beginning at the viral RNA 5' end with the transcription start site as +1, based on the HIV-1 HXB2 strain):

1. The R region (nt 1-96) is located at both the 5' and 3' ends of HIV-1 and marks the beginning of all viral transcripts at the 5' end. Besides facilitating strand transfer during reverse transcription, the R region also plays an important role in transcription. In the 5' R region, an RNA hairpin (nt 1-55) known as the trans-activating response elements (TAR), enhances transcription efficiency through interaction with viral protein Tat (99, 107, 114). The 3' R region provides the polyadenylation signal in the viral RNA transcript.

2. The U5 region of HIV-1 RNA (nt 97-181) engages in the initiation of reverse transcription and selection of reverse transcription primer (57, 59, 64, 66, 67, 127, 138-140). The primary transcript of HIV-1 contains a 3'-end U5 region that is later removed through the 3'-end processing. This processing is regulated by a GU-rich element within the 3' U5 region (71).

3. The PBS region of HIV-1 RNA (nt 182-199) is complementary to 3'-end 18 nucleotides of tRNA^Lys^3, which binds to the PBS region and serves as the primer for reverse transcription. The PBS region is the major determinant for the selection and use of a tRNA as the reverse transcription primer in HIV-1 (30, 65, 81, 128).

4. The gag leader sequence (GLS) (nt 200-335) contains three critical elements: the packaging signal (ψ) for incorporating genomic RNA into the virion, the dimerization site for facilitating the encapsidation of two genomic RNAs (26), and the major splice donor site for generating all the subgenomic spliced mRNAs.
5. The Rev response element (RRE) (nt 7321-7596) is the Rev binding site, located in the env coding region. RRE is required for the export of unspliced genomic RNA from the nucleus to the cytoplasm (37, 104).

6. The U3 region of HIV-1 genomic RNA was recently shown to be important for the strand transfer in reverse transcription (16).

HIV genomic RNA contains a 5' cap structure and 3' polyadenylate tail, closely resembling cellular mRNAs. HIV genomic RNA replicates through a DNA intermediate that integrates into the host chromosome, which is then transcribed into viral genomic and subgenomic RNAs. The viral DNA contains identical long terminal repeats (LTR), consisting of U3, R, and U5, at both ends. The 5' LTR and the GLS region of the viral DNA function in transcription regulation through interacting with numerous cellular transcription factors (103).

Viral Proteins

The gag, pol, and env open reading frames encode the Gag, Pol, and Env polyproteins, respectively, which are subsequently proteolyzed into mature proteins by proteases. The four Gag proteins, matrix (MA), capsid (CA), nucleocapsid (NC), and p6, and the two Env proteins, gp120 (SU) and gp41 (TM), are structural components of the virion. The three Pol proteins, protease (PR), reverse transcriptase (RT), and integrase (IN), provide essential enzymatic functions. HIV-1 encodes six additional proteins, often called accessory or auxiliary proteins, three of which (Vif, Vpr, Nef) are found in the virion. Although the other three (Tat, Rev, and Vpu) are not encapsidated into the virion, they provide essential functions in gene regulation and virion assembly (39).
Gag Proteins

**Matrix.** The MA protein is the N-terminal component of the Gag polyprotein. MA is important for directing Gag and Gag-Pol polyproteins to the plasma membrane for virus assembly. Trimerization of MA is important for virus assembly (39). MA may also help to incorporate Env into virion (32). During maturation of the virion, the 132-residue MA protein is cleaved from the polyproteins by the viral protease and lines the inner surface of the virion membrane. Two separate features of MA are involved in membrane targeting: the N-terminal myristate group and the basic residues of the membrane-binding surface of MA.

MA also facilitates the virus infection of nondividing cells. The mechanism for this function is not yet fully understood. One of the theories is that MA assists the nuclear transport of viral preintegration complex by providing a nuclear localization signal (17). MA may also be involved in directing unspliced viral RNA to the plasma membrane for virus assembly.

**Capsid.** CA is the center component of the Gag polyprotein and forms the core shell of the virion. The C-terminal domain of CA primarily functions in virus assembly and is important for the CA dimerization and Gag oligomerization (43). The N-terminal domain of CA is important for infectivity, apparently by participating in viral entry through its association with a cellular protein cyclophilin A (86).

**Nucleocapsid.** NC, the C-terminal component of the Gag polyprotein, is a 55-residue basic protein with two zinc finger domains. NC preferentially binds to the
packaging signal $\psi$ and delivers viral genomic RNA into the assembling virion (29, 31). With the capacity to nonspecifically bind single-stranded nucleic acids, NC coats the genomic RNA inside the virion core, which presumably helps to protect and compact the RNA. Additionally, NC promotes melting of RNA secondary structures and annealing of complementary sequences (108); therefore, it facilitates annealing of tRNA primer to the PBS, strand transfer during reverse transcription (18, 49, 54), integration of viral DNA (19), and perhaps also the dimerization of viral genomic RNA.

\textit{p6}. The 51-residue p6 protein is located at the C terminus of Gag. p6 specifically binds with Vpr, which helps to incorporate Vpr into the virion (75). p6 also contributes to efficient virion release (53).

\textit{Pol Proteins}

\textit{Protease}. PR is a component of Pol in the Gag-Pol polyprotein. Gag-Pol polyprotein is produced through ribosomal frameshifting following Gag translation. The amount of Gag-Pol is about 5-10% of Gag. After virus assembly, the virions released from the membrane surface are immature, containing Gag and Gag-Pol polyproteins that must be cleaved by PR to produce mature infectious virus. PR cleaves at several sites to produce the final MA, CA, NC, and p6 proteins from Gag and to produce PR, RT, and IN from Pol. The precise timing of PR cleavage is not clear but is presumably highly coordinated with virus assembly and budding. PR activity and cleavage rate may be modulated by many factors, including the Gag-Pol concentration, NC-RNA binding, p6 sequence, and the p2 spacer peptide located between CA and NC (39). PR functions as a
dimer with the catalytic site located at the dimer interface. PR has been a major target for drug design, and several PR inhibitors are in clinical use.

*Reverse transcriptase.* HIV RT is a heterodimer with both subunits (p66 and p51) derived from the Pol polyprotein. RT catalyzes reverse transcription to convert the viral RNA genome into a DNA duplex. RT catalyzes both RNA-dependent and DNA-dependent DNA polymerization and catalyzes cleavage of the RNA in RNA-DNA hybrids generated during reverse transcription. Both p66 and p51 subunits contain a polymerase domain composed of four subdomains called fingers, palm, thumb, and connection. These subdomains are arranged differently in the two subunits. In p66, they form an active-site cleft, whereas in p51 they form an inactive closed structure (74, 129). p66 also contains an RNase H domain, which catalyzes the degradation of RNA in RNA-DNA hybrids. The structures of RT have been studied extensively. Crystal structures of unliganded RT, an RT-DNA complex, and RT-inhibitor complexes have been determined (34, 51, 60, 74, 109, 113). The crystal structure of HIV-1 RT complexed with a DNA/DNA (primer/template) duplex indicates that the fingers, palm, and thumb of p66 form the nucleic acid binding channel. This channel accommodates about 18 base pairs (bp), with the 3’ end of the primer at the polymerase active site and the last base pair at the RNase H active site (52, 60). The relative positioning of the polymerase and RNase H active sites facilitates the coordination of RNase H degradation and DNA synthesis. A constant number of 18 bp of primer/template duplex is located between the two active sites (46). Most interactions between RT and the primer/template duplex occur around the polymerase active site and the RNase H active site.
RT initiates reverse transcription from the 3'-end of a tRNA primer annealed to the PBS region of the viral RNA genome. Mature RT interacts with tRNA^{lys3} with the similar efficiency as interacting with many other tRNAs (122). RT can use alternative tRNA primers to initiate reverse transcription when complementary primer binding sites are provided (30, 65, 81, 128). RT shows a kinetic transition from the initiation to elongation phase during the reaction, becoming highly processive during elongation (78). HIV-1 RT has a higher mutation rate (1/10^4 nucleotides/replication event) than RTs of other viruses such as avian myeloblastosis virus (~1/10^5 nucleotides/replication event) (106, 111). The high mutation rate of HIV-1 RT is due to the lack of 3'→5' exonuclease activity in RT and high pausing rate during HIV reverse transcription, along with other factors (7). RT has been a prime therapeutic target. Two classes of RT inhibitors, nucleoside analogs and nonnucleoside inhibitors, are in wide clinical use.

**Integrase.** Integrase (IN) is the C-terminal component of Pol polyprotein. IN catalyzes the integration of the viral genomic DNA into a host cell chromosome. The IN monomer can be divided into three domains, the N-terminal domain, the catalytic domain, and the C-terminal domain (110). The N-terminal domain contains a Zinc-binding site and DNA-binding structures similar to those in DNA-binding proteins. The catalytic domain contains a highly conserved motif that is crucial for the integration reactions. The C-terminal domain shows nonspecific DNA-binding activity. IN is active as an oligomer, probably a tetramer (110). IN catalyzes a series of reactions in the integration process, which is described later in the HIV-1 replication cycle section. No
exogenous energy source is needed for these reactions, whereas a metal ion (Mn$^{2+}$ or Mg$^{2+}$) is needed, presumably at the catalytic active site of IN (110).

**Env Proteins**

HIV-1 Env proteins are synthesized as a precursor that assembles into trimers, which are subsequently cleaved by a cellular protease to generate mature Env proteins, gp120 and gp41 (4).

**gp120 (SU).** The SU of viral Env, gp120, is located on the virion membrane surface. The binding of SU with host cell surface receptor CD4 is essential for virus entry. The SU-CD4 binding triggers conformational changes in Env that facilitate coreceptor binding and subsequent viral entry. These changes include the exposure of the V3 loop of SU that interacts with specific coreceptors, such as CXCR4, CCR5, and CCR3. The SU-coreceptor interaction is an essential factor for virus entry and an important determinant of viral tropism.

**gp41 (TM).** gp41, the TM of Env, is located in the viral membrane. The primary function of TM is to mediate fusion between the viral and cellular membranes. The transmembrane region of TM is important for fusion and anchoring Env in the membrane. The N-terminal fusion peptide of TM has been indicated to initiate fusion (22).
**Accessory Proteins**

HIV-1 encodes six so-called accessory or auxiliary proteins, which were initially thought to be dispensable for virus replication based on early in vitro studies. However, recent studies have demonstrated important functions for these accessory proteins in HIV-1 replication.

**Vif.** Vif is a 192-residue protein important for the infectivity of virions. Vif mutant viruses produced from certain cell types show altered core structures, reduced viral DNA synthesis, and defects in infectivity. Hence, Vif has been suggested to function during reverse transcription and to be involved in virus assembly and/or maturation (28).

**Vpr.** The 96-residue Vpr protein is important for the nuclear transport of the viral preintegration complex (PIC) following the virus entry. Vpr contains a nuclear localization signal that directs the nuclear transport of PICs in both dividing and nondividing cells (28). Vpr is incorporated into virions through its interaction with p6 in the Gag polyprotein, and Vpr later becomes associated with the nucleoprotein complexes through interacting with MA (118). Vpr also induces G2 cell cycle arrest. Additionally, Vpr interacts with tRNA\textsuperscript{Lys} synthetase to inhibit aminoacylation of tRNA\textsuperscript{Lys} (120).

**Vpu.** Vpu is an 81-residue integral membrane protein that facilitates virus assembly through promoting CD4 degradation. Vpu interacts with newly-synthesized CD4 in the endoplasmic reticulum. This interaction leads to degradation of CD4 and...
release of CD4-bound Env polyprotein for virus assembly. Vpu also stimulates virion release from the cell surface (47, 77).

Rev. Rev, like Tat and Vpu, is not found in the virion and is produced after the viral infection. Rev promotes the export of unspliced or single-spliced viral RNAs to the cytoplasm. Rev binds with the RRE site in the *erv*-coding region of these viral RNAs and delivers the bound RNAs to the cellular nuclear export machinery (37). The presence of both nuclear export and nuclear import signals in Rev allows it to shuttle continuously between the nucleus and cytoplasm (96). Rev may also inhibit viral RNA splicing by interfering with spliceosome assembly (23, 84). The functional domains of the 116-residue Rev include an arginine-rich domain that mediates specific RNA-binding and nuclear localization, a nuclear export domain, and a homomultimerization domain (104).

Tat. The 101-residue Tat protein promotes efficient transcription from the integrated provirus by enhancing the processivity of transcribing RNA polymerase. Unlike most transcription factors that bind to DNA sites, Tat binds to an RNA hairpin (TAR) near the 5' end of the nascent viral transcripts and increases production of viral mRNAs ∼100-fold (99, 114). Tat probably promotes the transition of RNA polymerase II from initiation phase (hypophosphorylated) to elongation phase (hyperphosphorylated) by recruiting or activating a Tat-associated cellular kinase (25, 100). The Tat-associated kinase increases the processivity of RNA polymerase II through hyperphosphorylating its carboxyl-terminal domain (CTD) (68, 107). Tat may also function in enhancing
transcription initiation and improving the accessibility of transcription factor-binding sites in the integrated DNA, probably through recruiting histone acetyltransferases to loosen the nucleosomal structures around the viral DNA (50, 93). In addition to its role in transcription, Tat also has nontranscriptional functions, which include activating T-lymphocytes and promoting efficient reverse transcription (107).

**Nef.** The 206-residue Nef appears to have a number of functions. The first described function of Nef is downregulating cellular CD4 and MHC class I molecules, similar to the function of Vpu (1, 44). Downregulation of CD4 probably benefits the virus at several levels, such as promoting efficient virus assembly and release. Downregulation of MHC molecules protects the virus from anti-HIV cytotoxic T-lymphocyte activity. Additionally, Nef also associates with numerous cellular proteins with signaling and cytoskeletal functions and is essential for HIV infectivity and pathogenesis (9, 92).

**HIV-1 Replication Cycle**

The HIV-1 replication cycle can be divided by the viral DNA integration step into early and late phases. The early phase of replication includes virus entry, reverse transcription, nuclear transport of the viral nucleoprotein complexes, and the integration of viral DNA. The late phase includes transcription of the viral DNA, viral gene expression, virion assembly, and virion release (Fig. 3).
FIG. 3. HIV-1 replication cycle. Major steps are described in the text.
**Virus Entry**

HIV-1 attachment to target cells is a multistep process. It was demonstrated recently that the direct binding of cyclophilin A in the viral membrane to the cell surface heparans represents the essential initial step of HIV-1 attachment (116). This initial attachment is followed by interaction between the viral gp120 and the cell surface CD4 antigen (72, 94). As a member of the immunoglobulin superfamily, CD4 is expressed in a number of cell types, including T-helper cells, macrophages, monocytes, dendritic cells, and brain microglia. These are the main target cells for HIV in vivo. gp120-CD4 binding induces conformational changes in the gp120, which lead to the formation or exposure of the binding site for the coreceptors of virus entry (135). Subsequently, the CD4-bound gp120 interacts with one of the coreceptors, which are chemokine receptors or related orphans (12). CXCR4 and CCR5 are the principal coreceptors for T-cell line-tropic and macrophage-tropic HIV-1 isolates, respectively. Additional chemokine receptors with coreceptor activity have also been identified. The different use of chemokine receptors by different HIV-1 isolates and the different expression patterns of these coreceptors in different cell types primarily account for the viral tropism (12).

The binding of the gp120-CD4 complex with the coreceptor promotes additional conformational changes in the gp120 and gp41 complex. These changes result in the insertion of the hydrophobic gp41 NH2-terminus (the "fusion peptide") into the membrane of the target cell (21, 130). Eventually, these events lead to direct fusion of the viral and cell membranes. The viral nucleocapsid is then released into the cytoplasm of the target cell.
Reverse Transcription

Reverse transcription is a process to convert RNA into DNA. RT provides all the enzymatic functions that are required for reverse transcription: a DNA polymerase that can use either RNA or DNA as a template and a ribonuclease H (RNase H) that degrades the RNA moiety of RNA-DNA hybrids. The HIV-1 reverse transcription process can be divided into five major steps: reverse transcription initiation, first strand transfer, minus-strand synthesis, plus-strand synthesis, and second strand transfer and continued DNA synthesis. These major steps are illustrated in Fig. 4 and described as follows.

1. The 3’ 18 nucleotides of tRNA<sub>Lys</sub><sup>3</sup>, the natural primer for HIV-1 reverse transcription, is complementary to the PBS of the viral RNA genome. Prior to reverse transcription, tRNA<sub>Lys</sub><sup>3</sup> is annealed to the PBS downstream of the U5 region. Reverse transcription initiates from the 3’ end of the annealed tRNA<sub>Lys</sub><sup>3</sup> and proceeds until it reaches the 5’ end of the viral RNA genome. The resultant DNA intermediate is called minus-strand strong-stop DNA (-sssDNA).

The timing for the initiation of HIV reverse transcription remains to be solved. Several-nucleotide-long viral DNA oligomers have been identified inside HIV-1 virions. This might suggest that HIV-1 reverse transcription is initiated before virus entry, with the remainder of the reverse transcription taking place after the virus entry (6, 56, 101). Conversely, the DNA oligomers in the virions might be a byproduct that is unnecessary for the virus life cycle (6).

2. Following the degradation of the RNA strand of the RNA : -sssDNA duplex, the first strand transfer takes place, resulting in the annealing of -sssDNA to the 3’ end of the viral genomic RNA. This transfer is mediated by the complementarity
FIG. 4. The process of HIV-1 reverse transcription.
between the R region of the nascent −sssDNA and the plus-sense R region at the 3' end of the viral RNA genome. The transfer is facilitated by NC protein and by the interaction between the tRNA primer and the U3 sequence of the viral RNA (16). Since the virus contains two copies of the genomic RNA, the strand transfer could occur either as an intramolecular or intermolecular event, possibly with a similar frequency (123).

3. After the first strand transfer, minus-strand DNA synthesis resumes and is followed by the degradation of the template strand by RNase H.

4. The viral RNA genome contains two short polypurine tracts (PPTs) that are relatively resistant to the RNase H digestion. Both PPTs function as primers for plus-strand DNA synthesis. The 3' PPT is located near the 3' end of the viral RNA (3' PPT), priming the synthesis of the so-called upstream segment (U+). The center PPT (cPPT) is in the center of the viral RNA genome, priming the synthesis of the downstream segment (D+). U+ DNA synthesis proceeds until the 3'-end 18 nucleotides of the tRNA primer is reverse transcribed. The resultant DNA is also called the plus-strand strong-stop DNA (+sssDNA). Presumably, the termination of +sssDNA is due to the encounter of the first modified tRNA base located 19 nucleotides upstream from the tRNA 3' end by RT (11). Subsequently, RNase H removes the tRNA primer and the PPTs from the DNA.

5. The second strand transfer, which is mediated by the annealing of the complementary PBS segments in the +sssDNA and minus-strand DNA, results in the formation of a circular DNA intermediate. The second strand transfer enables the plus- and minus-strand DNA syntheses to continue, with the plus and minus strands of DNA serving as templates for each other. Synthesis of the U+ DNA proceeds until it reaches a region called the central termination sequence (CTS), located ~100 nt downstream of the
5' end of the D+ DNA (24). As a result, the final DNA duplex contains a ~99 nt plus-stand overlap in its center, called the central DNA flap.

Reverse transcription generates a linear DNA duplex from the viral RNA genome. This DNA form of the viral genome contains two LTRs at the ends; each consists of U3, R, and U5. Therefore, the HIV DNA is longer than its RNA genome at both ends, because of the duplication of the U5 and U3 regions.

**Nuclear Import**

After reverse transcription, a large subviral nucleoprotein complex, known as the preintegration complex, needs to be imported into the nucleus for integration. The HIV PIC contains the viral DNA, IN, RT, MA, Vpr, NC, and possibly PR (38). A number of cellular proteins, including importin-α, are also associated with PICs. IN, Vpr, and MA have been implicated in mediating the nuclear import of PICs. Both IN and Vpr provide nuclear localization signals and interact with cellular importin-α, which directs the PIC to the cellular pathway of nuclear import (42, 105). The role of MA in nuclear import remains controversial and unsolved (38).

Recently, Zennou and colleagues identified the central DNA flap of the viral DNA as the cis-determinant for the nuclear import of HIV-1 DNA (137). The central DNA flap is essential for HIV-1 DNA nuclear import. Mutant viral DNA lacking the DNA flap accumulates at the vicinity of nuclear membrane as unintegrated linear DNA. The mechanism by which the central DNA flap facilitates nuclear import remains to be determined.
Integration

Integration of the viral DNA into the host cell genome is an essential step in the life cycle of HIV. Integration stabilizes viral DNA against degradation and enables it to genetically transmit as an integrated element of the host genome. Before integration, the linear viral DNA is associated with viral and cellular proteins in the PIC. The PIC alone is capable of integrating viral DNA, as demonstrated in vitro (35, 36). The integration process for HIV is summarized as follows (15):

1. After the completion of viral DNA synthesis, IN removes two bases from each of the 3' termini of the viral DNA duplex to generate a recessed CA-OH end with free 3'-OH group.

2. The IN-viral DNA complex targets a host DNA site through an unknown mechanism. Potential preference in the target site selection for HIV DNA integration remains to be determined. It has been proposed that integration is directed to sites favorable for transcription (63). The integrase catalyzes the attack of two phosphodiester bonds on the target DNA by the 3' -OH groups of the viral DNA. These two phosphodiester bonds are five bases away from each other, located on opposite strands but on the same face of the target DNA double-helix. New bonds are formed to join the viral DNA 3' ends with the target DNA.

3. The mismatched nucleotides at the viral DNA 5' ends are removed, and the gaps flanking the viral DNA are filled by extending the free 3' ends of host DNA. The resultant 5' and 3' ends are then ligated. The enzyme(s) involved in this step remains to be determined. The proposed candidates include IN, RT, and cellular enzymes. After the
completion of integration, the viral DNA (provirus) is flanked by 5-bp direct repeats of the target site DNA.

Unintegrated viral DNA in circular forms has been detected in the nuclei of HIV-1 infected cells. These circular viral DNAs appear to be by-products of abortive infections and do not significantly contribute to the virus replication (15).

**Gene Expression**

After integration, the integrated HIV DNA mimics a cellular gene and relies almost entirely on the host cell machinery for gene expression. Cellular RNA polymerase II catalyzes the viral RNA transcription, which initiates at the U3/R border of the 5' LTR and terminates around the R/U5 border of the 3' LTR. The LTRs, in particular the U3 regions, contain numerous binding sites for a wide range of cellular transcription factors to regulate the transcription efficiency (103).

The levels of the viral transcripts are also regulated by viral factors. Early after integration, only low levels of viral RNA are generated because of the poor processivity of RNA polymerase II. Once viral Tat protein is produced, the transcription of viral RNAs is enhanced to a much higher level. Tat binds with the TAR element near the viral RNA 5' end to increase the processivity of the transcribing RNA polymerase II (68, 107). Tat may also facilitate the binding of transcription factors with the LTRs to enhance the transcription initiation (50, 93).

The nascent viral transcripts are subject to the same processing events as cellular RNAs, including the 5' cap addition, 3' end polyadenylation, and splicing, all via cellular RNA processing machinery. Unlike the splicing of most cellular mRNAs, in which
introns are spliced out completely, splicing of the viral RNA is regulated to maintain a proper ratio between the spliced and unspliced RNAs. The unspliced RNA serves as the genomic RNA as well as mRNA to generate Gag and Pol. The spliced viral RNAs function as mRNAs for Env and accessory proteins. The ratio between them is regulated by Rev. Rev promotes the export of unspliced or single-spliced viral RNAs to cytoplasm (37) and may also inhibit viral RNA splicing by interfering with spliceosome assembly (14, 84). In the early stage of gene expression, multiply spliced viral RNAs are generated and exported to the cytoplasm through the normal mRNA export pathway. Once Rev is produced from these early mRNAs, it shuttles between nucleus and cytoplasm to promote the export of intron-containing viral RNAs (104).

The translation of the Gag-Pol polyprotein from the unspliced viral RNA is a result of frameshifting. About 5-10% of the time ribosomes slip back 1 nt (-1 frameshift) during translation of Gag, which results in bypass of the gag termination codon and continued translation of the overlapping pol reading frame. Frameshifting serves as a simple strategy to control the proper ratio of Gag and Gag-Pol polyproteins. This strategy also provides an easy way to incorporate the essential enzymes of Pol into the virion during assembly. Gag proteins are subject to additional modifications during or after their synthesis, which include myristylation and phosphorylation (121).

Env is produced via the same synthesis and modification pathways as cellular surface proteins. Vpu helps to free Env oligomers from binding with CD4 in the endoplasmic reticulum and therefore facilitates Env transport. Newly-synthesized Env is cleaved into subunit SU and TM by a cellular protease in the Golgi apparatus before being transported to the cell surface (121).
Virus Assembly and Release

The Gag polyprotein is the driving force of virus particle assembly and release. The Gag-Gag interaction domain within the NC sequence mediates the interactions among Gag molecules. The membrane-binding domain of Gag, located within the MA sequence, is essential for membrane binding and budding. The so-called L domain within the p6 sequence is involved in the last step of budding (121). The Gag portion of the Gag-Pol polyprotein directs the Gag-Pol to the assembly site. Viral genomic RNA is specifically packaged into the virion through the recognition of the packaging signal by Gag (13, 87). It is unclear how HIV incorporates two genomic RNA molecules. The dimerization initiation site in the viral RNA might promote the encapsidation of viral RNA dimer (27). However, whether the dimerization occurs before virus release remains to be determined.

Like other retroviruses, HIV-1 virions contain a substantial amount of cellular tRNAs. These tRNAs do not represent a random sampling of the cellular pool; tRNA^{Lys1,2,3} are greatly enriched. The mechanism for the selective packaging of tRNAs remains to be solved.

HIV accessory proteins are also involved in virus assembly and maturation. Vif has been suggested to function in virus assembly and/or maturation through a not yet defined mechanism (28). Vpu enhances the release of viral particles (47, 77), and Nef downregulates cell surface CD4 to prevent progeny virions from being trapped at the cell surface.
tRNA and Its Role in Reverse Transcription

tRNAs are small RNA molecules present in all organisms. tRNAs are the key adapters in translating the language of genetic code into the language of polypeptides. All cells contain many species of tRNAs, at least one for each of the 20 amino acids. This section reviews the general properties of tRNA and its involvement in reverse transcription in HIV-1 as well as in several other retroviruses and retrotransposons.

**General Properties of tRNA**

**Primary and Secondary Structures of tRNA**

Most known tRNAs have ~76 nt and may be arranged in the so-called cloverleaf secondary structure (Fig. 5A). The tRNA cloverleaf generally can be divided into five regions: the acceptor stem, D stem-loop, anticodon stem-loop, variable loop, and TΨC stem-loop. The acceptor stem typically contains 7 bp and carries the amino acid residue at its 3'-terminal OH group. The D stem-loop frequently contains the modified base dihydouridine (D). The 5-bp anticodon stem ends in a loop that contains the anticodon, the three bases complementary to the codon specifying the tRNA. The variable loop has the greatest variability among tRNAs. The TΨC stem-loop usually contains three bases, TΨC. There are 15 invariant positions (always the same base) and 8 semi-invariant positions (only a purine or only a pyrimidine) that are primarily located in the loop regions (Fig. 5A). All tRNAs terminate in CCA with a free 3'-OH group (124).

One of the unique features of tRNAs is the presence of modified bases in up to 60 different positions. tRNA modifications have important biological functions, such as
FIG. 5. The structure of the tRNA. (A) The cloverleaf secondary structure of tRNA. Circles connected by dashes represent bases involved in basepairing. Invariant and semi-invariant positions are indicated. R and Y represent invariant purines and pyrimidines, respectively. Ψ specifies pseudouracil. The starred nucleotides are often modified. The dashed region in the variable loop contains different numbers of nucleotides in various tRNAs. (B) A simplified representation of the three-dimensional structure of yeast tRNA\textsuperscript{Phe}. Colors are used to illustrate the positions of the stem-loops in the L shaped molecule. The acceptor stem and TΨC stem-loop are red, the D stem-loop is blue, the variable loop is gray, and the anticodon stem-loop is yellow.
improving the efficiency and accuracy of translation. However, most of these modifications are not essential for cell viability nor for the tRNA structural integrity.

**Tertiary Structure of tRNA**

The three-dimensional structure of tRNA is best characterized in yeast tRNA^Phe^ (70, 112) (Fig. 5B). The tertiary structures of three other tRNAs have also been elucidated (124). These tRNAs all form a similar L-shape structure, which can be divided into two halves: the top half consists of the acceptor stem and TΨC stem-loop; the bottom half consists of the D stem-loop, anticodon stem-loop, and variable loop. The tertiary structure of yeast tRNA^Phe^ is maintained through large numbers of intramolecular associations, including stacking interactions and tertiary base-pairing interactions (70, 112).

**Biosynthesis of tRNA**

For relatively small molecules, the biosynthesis of functional, mature tRNAs is an amazingly complicated process (134). The initial transcripts of tRNA genes produced by RNA polymerase III are precursor molecules, which undergo enormous changes, including end maturation, intron splicing, nucleotide modification, and CCA addition (for eukaryotic tRNAs). Most studies on the processing of eukaryotic tRNAs were done in yeast systems. Since almost all the components identified in yeast tRNA processing have counterparts in higher eukaryotic cells, tRNA processing in eukaryotes is expected to be highly conserved.
All newly synthesized pre-tRNAs contain 5' leader and 3' trailer sequences that must be removed. In eukaryotes, a highly abundant nuclear protein La binds to all newly synthesized pre-tRNAs. The La protein-bound pre-tRNAs are the substrates for RNase P, which removes the 5' leader sequence (134). An unidentified endonuclease removes the 3' trailer. For a subset of pre-tRNAs that contains introns, splicing usually occurs after the end maturation. Both the end maturation and splicing take place in the nucleus. Base modification of tRNAs occurs in both nucleus and cytoplasm. Some modifications are added to newly synthesized pre-tRNAs, some are added to end-matured but intron-containing pre-tRNAs, whereas others occur after the intron-splicing. Before leaving the nucleus, CCA is added to the 3' termini of eukaryotic tRNAs. The CCA-adding enzyme also functions in the cytoplasm to repair the damaged CCA-termini of tRNAs (133).

Mature tRNAs are exported to cytoplasm by exportin-t together with RanGTPase. Exportin-t-RanGTPase selectively exports mature tRNAs but not immature or misfolded tRNAs and therefore contributes to tRNA quality control (8, 76). It had long been believed that tRNA aminoacylation, the attachment of amino acids to tRNAs, occurred after tRNAs were exported to cytoplasm. Recently Lund and Dahlberg demonstrated that, in Xenopus oocytes, tRNA aminoacylation also occurs in the nucleus (88). Furthermore, aminoacylation facilitates the export of tRNAs to cytoplasm.

**tRNA Aminoacylation**

tRNA aminoacylation is catalyzed by aminoacyl-tRNA synthetase. At least one aminoacyl-tRNA synthetase is needed for each amino acid. Despite the similarity of the aminoacylation reactions and despite the structural similarity of all tRNAs, the
aminoacyl-tRNA synthetases are a group of enzymes with diverse subunit structures and little sequence similarity among synthetases for different amino acids. Molecular signals in a tRNA that trigger its specific aminoacylation by the cognate synthetase are usually referred to as tRNA identity determinants (elements). Identity determinants lie predominantly at two regions: the acceptor stem and the anticodon loop (45). The so-called discriminator nucleotide N73 and the three anticodon nucleotides are the major identity determinants for most tRNAs. For example, the identity of yeast tRNA^{Phe} is governed by five nucleotides: A73, G20, and the three anticodon nucleotides (115). Nearly all determinants are in direct contact with the cognate synthetases. After the aminoacylation, mature tRNAs become fully functional, and are subsequently delivered to ribosomes by elongation factor EF-1 to participate in translation.

Based on studies in a permeabilized CHO cell system, Deutscher and co-workers have suggested that endogenous tRNAs in the cytoplasm are never free of the protein synthetic machinery as translation proceeds. During repeated cycles of translation, aminoacyl-tRNAs are directly transferred from the aminoacyl-tRNA synthetases to EF-1 to ribosomes and then are transferred back to the synthetases without dissociating into the cellular fluid (119).

**tRNA as Reverse Transcription Primer**

Cellular tRNAs function as primers for reverse transcription in both retroviruses and LTR retrotransposons. Different retroviruses and retrotransposons select different tRNAs as primers, examples of which are illustrated in Table 1 (91). tRNA^{Trp}, tRNA^{Lys},
and tRNA^{Pro} are the best-studied reverse transcription primers for retroviruses (see Table 1). Lentiviruses, including HIV, use tRNA^{Lys,3} as the primer.

**TABLE 1. Examples of the tRNAs used by retroviruses and retrotransposons as reverse transcription primers**

<table>
<thead>
<tr>
<th>Retrovirus or retrotransposon</th>
<th>tRNA primer</th>
<th>Length of primer binding site (bases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian sarcoma and leucosis virus group</td>
<td>tRNA^{Trp}</td>
<td>18</td>
</tr>
<tr>
<td>Moloney murine leukemia virus</td>
<td>tRNA^{Pro}</td>
<td>18</td>
</tr>
<tr>
<td>Lentiviruses and mouse mammary tumor virus</td>
<td>tRNA^{Lys,3}</td>
<td>18</td>
</tr>
<tr>
<td>Human foamy virus and Mason-Pfizer monkey virus</td>
<td>tRNA^{Lys,2}</td>
<td>18</td>
</tr>
<tr>
<td><strong>Yeast transposons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ty1 and Ty2</td>
<td>tRNA^{Met-i}</td>
<td>10</td>
</tr>
<tr>
<td>Ty3</td>
<td>tRNA^{Met-i}</td>
<td>8</td>
</tr>
<tr>
<td>Ty4</td>
<td>tRNA^{Asn}</td>
<td>18</td>
</tr>
<tr>
<td><strong>Drosophila transposons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gypsy</td>
<td>tRNA^{Lys}</td>
<td>11</td>
</tr>
<tr>
<td>copia</td>
<td>tRNA^{Met-i} fragment</td>
<td>18</td>
</tr>
</tbody>
</table>

Before a tRNA can be used to initiate reverse transcription, it needs to be specifically selected out of the cellular tRNA pool, packaged into the virions (retroviruses) or virus-like particles (VLP) (retrotransposons), and positioned onto the primer binding site of the genomic RNA to form an initiation complex together with RT and possibly other proteins. This complicated process is not yet fully understood. Presumably, it involves interactions of tRNA with a number of viral factors. RT, NC,
and the RNA genome of retroviruses/retrotransposons have been identified to interact with their tRNA primers.

*tRNA-RT Interaction*

HIV-1 RT interacts with the tRNA primer in vitro regardless of the absence or presence of an RNA template. The anticodon loop, TΨC loop, and D loop of tRNA\textsuperscript{Lys,3} have all been suggested to interact with HIV-1 RT in vitro (10, 97, 117, 132). Additionally, the 5' end of tRNA\textsuperscript{Lys,3} was shown to specifically interact with the C-terminal portion of HIV-1 RT in vitro (97). However, the interaction between RT and tRNA\textsuperscript{Lys,3} also appears to be non-specific, since RT can bind with many tRNAs, including tRNA\textsuperscript{Lys,3}, with the same efficiency (122). The involvement of the tRNA-RT interaction in the selection and use of tRNA primer in HIV-1 remains unsolved.

*tRNA-NC Interaction*

NC binds with tRNAs as well as other RNAs through its nonspecific RNA binding ability. NC facilitates the initiation and first strand transfer of reverse transcription by promoting tRNA-viral RNA annealing (16, 18, 49). NC promotes unwinding of the tRNA primer and its subsequent annealing to the complementary sequence. The unwinding of tRNA\textsuperscript{Lys,3} by HIV-1 NC requires the presence of a viral RNA genome (20, 48).
tRNA-Viral RNA Interaction

Extensive interactions occur between the retroviral RNA genome and its cognate tRNA primer. In addition to the tRNA 3'‐end nucleotides and PBS interaction, each retrovirus or retrotransposon has apparently developed specific features in its genomic RNA‐tRNA interaction to optimize the primer selection and use. The anticodon of tRNA\textsubscript{Lys}^3 interacts with a so‐called A‐loop region upstream of the HIV‐1 PBS in vitro (57, 58). This interaction is unique for HIV‐1 and is important for efficient reverse transcription in vivo (66, 67, 82, 127). For avian sarcoma and leucosis viruses, the U5 sequence of their RNA genomes interacts with the T\PsiC loop of the cognate tRNA primer (tRNA\textsubscript{Tri}^p), an interaction that is essential for the initiation of reverse transcription in vitro (2, 3, 98). Although similar complementarity is present between the U5 sequence of HIV‐1 and murine leukemia virus (MLV) and the T\PsiC stem‐loop of their cognate tRNA primers (tRNA\textsubscript{Lys}^3 and tRNA\textsubscript{Pro}^p, respectively), there is no evidence that the interactions actually take place in HIV‐1 and MLV. In the retrotransposon Ty1, the T\PsiC and D stem‐loops of tRNA\textsubscript{Met}^i interact with the PBS downstream sequence of the Ty1 RNA genome. This interaction is critical for the initiation of Ty1 reverse transcription in vivo (40, 69).

Interactions between the HIV‐1 RNA genome and the tRNA primer is not yet fully understood. Additional interactions probably remain to be identified. Recently, the anticodon stem of tRNA\textsubscript{Lys}^3 was shown to interact with the U3 sequence of the HIV‐1 RNA genome, which facilitates the first strand transfer of reverse transcription in vitro (16).
Encapsidation of tRNA Primer

A variety of cellular tRNAs, around 50 molecules, are packaged into the virion or VLP during virus assembly (126). These tRNAs do not represent a random sampling of the cellular pool; in HIV-1, tRNA\textsuperscript{Lys,3} and tRNA\textsuperscript{Lys,2} are greatly enriched (91). The mechanism for the selective encapsidation of tRNAs is not yet fully understood. In the retrotransposon Ty1, the encapsidation of the tRNA primer (tRNA\textsuperscript{Met,i}) requires the PBS and surrounding elements of the Ty1 RNA genome (131), whereas in HIV-1, the absence of the viral RNA genome apparently has no detectable impact on tRNA encapsidation in HIV-1 (62, 90). Instead, the RT sequence in the Gag-Pol polyprotein is required for the selective enrichment of the tRNA primer in retroviruses, including MLV and HIV-1 (80, 90, 91). The NC portion of HIV-1 Gag/Gag-Pol may also be involved in the encapsidation of tRNA\textsuperscript{Lys} (55). However, the interactions of RT and NC with tRNA\textsuperscript{Lys} appear to be non-specific; both can bind with many tRNAs with the same efficiency (95, 122), making it questionable that RT and NC are responsible for the selective enrichment of tRNA\textsuperscript{Lys,3}. Furthermore, the enrichment of certain tRNAs and the selective packaging of the actual tRNA primer molecule might be two separate events, since the lack of tRNA primer enrichment in the virion does not necessarily affect the selection and use of the actual tRNA primer molecule in vivo (41, 80, 138). In the absence of RT, MLV virions still contain the tRNA primer (tRNA\textsuperscript{Pro}) annealed to the PBS of the viral genome (41, 80). For a mutant HIV-1 using tRNA\textsuperscript{His} as the primer, tRNA\textsuperscript{His} within the virions is not enriched and stays as a minor population in the virion tRNA pool (138). Further studies are clearly required to understand the mechanism of tRNA primer selection and packaging.
The Focus of the Dissertation Research

The selection and use of tRNA primer for HIV-1 reverse transcription in vivo is a complex process that involves multiple factors. The mechanisms underlying this process remain to be solved. The role of viral factors, mainly RT, NC, and the viral RNA genome, has been the focus of most studies in this area. Little is known about the determinants within the tRNA for its selection and use in reverse transcription of HIV or other retroviruses. Studies in yeast retrotransposon Ty1 and Ty3 have identified elements of their tRNA primer (tRNA\textsuperscript{Met-i}) that are critical for reverse transcription in vivo (40, 69). These studies took advantage of the powerful genetic capabilities of the yeast system to manipulate the sequence of tRNA\textsuperscript{Met-i}. It would be rather difficult to carry out a similar study on HIV-1, because of the complexity in manipulating endogenous tRNA\textsuperscript{Lys,i} in mammalian cells. A substitute would be to address the question using a simplified in vitro system. However, in vitro systems have their limitations in revealing the in vivo situation in the initiation of reverse transcription of retroviruses. The goal of this research is to study essential elements of a tRNA primer for HIV-1 replication by establishing an alternative in vivo system.

The first paper in this dissertation focuses on the primer use and replication efficiency of HIV-1 mutants with alternative PBS in a single round of infection. Additionally, the development of an in vivo complementation system was described, in which a mutant HIV-1 (psHIV-Phe) relied on an exogenous tRNA as primer for reverse transcription. psHIV-Phe contains a PBS complementary to yeast tRNA\textsuperscript{Phe}, which is not infectious unless yeast tRNA\textsuperscript{Phe} is provided during the virus production. This system allows manipulation of the tRNA primer for reverse transcription of psHIV-Phe.
The second paper presents the studies on elements of tRNA\textsuperscript{Phe} important for psHIV-Phe infectivity. A series of yeast tRNA\textsuperscript{Phe} mutants were tested for their capacity to rescue the psHIV-Phe virus in our complementation system. The results demonstrate that the tRNA acceptor stem, TΨC stem-loop, and anticodon stem-loop are important for psHIV-Phe infectivity, whereas the D stem-loop is not required for the virus replication.

Studies described in the third paper further dissected the role of the acceptor stem and TΨC stem-loop of tRNA\textsuperscript{Phe} in primer selection and use. The results indicate that, for the infectivity of psHIV-Phe, the 5' end of the tRNA is critical, whereas the 3' end ACCA sequence is not required. Certain single-nucleotide changes in the tRNA TΨC stem-loop are detrimental to the virus infectivity. This result led to the hypothesis that the TΨC stem-loop sequence specifically interacts with HIV-1 RNA genome to facilitate the selection and use of the tRNA primer.

Taken together, the results of these studies demonstrate the importance of specific elements of the tRNA primer for HIV-1 replication. This information will help to understand the mechanism of tRNA primer selection and use in HIV-1 and will help to define new targets for interruption of HIV-1 replication.
COMPLEMENTARITY BETWEEN 3’ TERMINAL NUCLEOTIDES OF tRNA AND PRIMER BINDING SITE IS A MAJOR DETERMINANT FOR SELECTION OF THE tRNA PRIMER USED FOR INITIATION OF HIV-1 REVERSE TRANSCRIPTION

by

QIN YU AND CASEY D. MORROW

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ABSTRACT

The initiation of reverse transcription of human immunodeficiency virus type 1 (HIV-1) exclusively utilizes tRNA\textsuperscript{Lys,3} as primer. Previous studies have shown that HIV-1 could use alternative tRNAs, such as tRNA\textsuperscript{Ile} or tRNA\textsuperscript{His}, to initiate reverse transcription only if the primer binding site (PBS) was made complementary to the 3' terminal 18 nucleotides of the cognate tRNA. However, upon in vitro culture, the viruses with a PBS complementary to the alternative tRNAs rapidly reverted to generate a PBS complementary to tRNA\textsuperscript{Lys,3}. To investigate the process of reversion, we have constructed defective proviral genomes that contain a PBS complementary to tRNA\textsuperscript{Ile} or tRNA\textsuperscript{His}. The genomes contain the gene for xanthine-guanosine phosphoribosyl transferase (gpt) in place of env. Cotransfection of these proviral genomes with a plasmid encoding vesicular stomatitis virus G protein (VSV-G) results in viruses that undergo a single round of HIV-1 infection; successful infections are scored as cells resistant to the drug mycophenolic acid. Using this single-round infection system, we demonstrated that HIV-1 with a PBS complementary to tRNA\textsuperscript{Ile} or tRNA\textsuperscript{His} is three- to fivefold less efficient in replication as measured by production of drug-resistant cell colonies compared to the wild-type virus. These viruses predominantly used the cognate tRNA as primer in their initial round of replication, although we did obtain a single cell colony in which the PBS was complementary to tRNA\textsuperscript{Lys,3}. Using an HIV-1 provirus with a PBS complementary to yeast tRNA\textsuperscript{Phe}, we established a single-round infection system in which the infectivity of this mutant HIV-1 relies on transfected yeast tRNA\textsuperscript{Phe}. The results of our studies suggest that the mechanism for selection of the tRNA primer for initiation of reverse
transcription rely primarily on the complementarity between the tRNA primer and the PBS.

INTRODUCTION

The initiation of retroviral reverse transcription occurs at a region in the viral RNA genome designated as the primer binding site (PBS). The 18 nucleotides comprising the PBS are complementary to the 3' terminal 18 nucleotides of the tRNA primer that is used for initiation of reverse transcription. The reverse transcriptase (RT) extends the 3' OH of the cellular tRNA molecule bound to the PBS. Following completion of reverse transcription, the DNA copy of the viral genome, termed the provirus, is integrated into the host cell chromosome (4, 13). During the plus strand synthesis step in reverse transcription, the RT copies the tRNA primer to generate a copy of the PBS (18). Thus, the DNA sequence of the PBS from the integrated provirus reflects the tRNA primer used for initiation of reverse transcription.

In general, retroviruses use different, but specific, tRNAs as primers for initiation of reverse transcription (9). For example, human immunodeficiency virus (HIV) exclusively uses tRNA^{Lys3} as the primer (14, 15). In contrast, human T-cell leukemia virus uses tRNA^{Lys1,2}, rather than tRNA^{Lys3}, for initiation of reverse transcription; murine leukemia virus utilizes tRNA^{Pro} (12, 17) while avian leukosis virus utilizes tRNA^{Trp} (23, 24). Previous studies from this laboratory and others have found that alteration of the PBS from HIV-1 to be complementary to tRNAs other than tRNA^{Lys3} resulted in viruses using these alternative tRNAs for initiation of reverse transcription (3, 5, 7, 22). Thus.
there appears to be a great deal of flexibility with respect to different tRNAs which can be used for initiation of reverse transcription. Although HIV-1 can use different tRNAs for initiation of reverse transcription when the PBS was altered so as to be complementary to those different tRNAs, HIV-1 reverted to utilize the wild-type tRNA primer (tRNA^{Lys,3}) after limited in vitro culture. The reason why these viruses reverted back to utilize tRNA^{Lys,3} as opposed to another tRNA for initiation of reverse transcription is not clear. Using an in vitro reconstituted reverse transcription system, it has been shown that tRNA^{Lys,3} is the most efficiently used primer for initiation of reverse transcription (10). Previous studies from this laboratory have shown that nucleotide changes in the 5' unique region (U5) in combination with the PBS could affect the selection of tRNA used for initiation of reverse transcription (5, 6, 19).

To further delineate the mechanism by which HIV-1 selects the tRNA^{Lys,3} for reverse transcription, we have developed a system that allows examination of the status of the PBS following a single round of infection (26). For these studies, a defective provirus was used in which the envelope gene has been substituted with a gene encoding xanthine-guanosine phosphoribosyl transferase (gpt) (11). Complementation of this defective genome with a suitable glycoprotein (e.g., VSV-G) allows a single-round infection of this virus. Successful reverse transcription events are monitored by the production of cell clones that are resistant to mycophenolic acid. We have used this system to compare the primer tRNA selection for reverse transcription of viruses with a PBS complementary to alternative tRNAs, tRNA^{His} or tRNA^{Hle}, with that of the wild-type virus. The results of our study clearly show that viruses with a PBS complementary to these alternative

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tRNAs predominately used these tRNAs for the first round of infection. The capacity of the viruses with a PBS complementary to an alternative tRNA to induce drug resistant colonies though was less than that of the wild-type virus. To exploit this result, we have also utilized an HIV-1 provirus with a PBS complementary to yeast tRNA\(^{\text{Phe}}\). Pseudotyped virus with a PBS complementary to yeast tRNA\(^{\text{Phe}}\) was not capable of generating drug resistant colonies unless yeast tRNA\(^{\text{Phe}}\) was supplied in trans. The results of our studies then are discussed with respect to the mechanism by which tRNA is selected as a primer for reverse transcription.

**MATERIALS AND METHODS**

**Materials**

All chemicals were purchased from Sigma Chemical Co. unless otherwise specified. Restriction endonucleases were obtained from New England Biolabs. The *Taq* polymerase and reagents for PCR, the tissue culture medium and reagents, the synthetic oligonucleotides used for PCR and DNA sequencing, and lipofectin were purchased from GIBCO BRL. The calcium-phosphate transfection kit was obtained from Qiagen. The enzyme-linked immunosorbent assays (ELISAs) for p24 antigen were obtained from Coulter Laboratories.

**Tissue Culture and Cell Lines**

293T cells and HeLa H1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum and 1% antibiotics in 37°C incubator supplied with 5% CO\(_2\).
Construction of HIV-1 Proviruses

pHXB2\textit{gpt}, a plasmid encoding the full-length HIV-1 provirus, with modified PBS were used for plasmid construction: pHXB2(Ile) contains a PBS region complementary to the 3'-terminal 18 nucleotide sequence of tRNA\text{Ile}; pHXB2(His) contains a PBS region complementary to the 3'-terminal 18 nucleotide sequence of tRNA\text{His} (22); pHXB2(Phe) contains a PBS region complementary to the 3'-terminal 18 nucleotide sequence of yeast tRNA\text{Phe} (21). pHXB2\textit{gpt} with a wild-type PBS is referred as pHXB2(wt).

Plasmid HIV-\textit{gpt} containing the mutant HIV-1 proviral genome was kindly provided by Dr. Dan Littman (11). HIV-\textit{gpt} has a 1.2-kb deletion in \textit{env} gene. A 1.1-kb fragment containing the simian virus 40 (SV40) promoter and the xanthine-guanosine phosphoribosyl transferase (\textit{gpt}) gene was inserted at the \textit{env} site (11). This \textit{env} deletion and \textit{gpt} insertion were transferred into pHXB2(wt), pHXB2(Ile), pHXB2(His) and pHXB2(Phe) by substituting the \textit{SalI-XbaI} fragment of these pHXB2 plasmids with the \textit{SalI-XbaI} fragment from HIV-\textit{gpt}. The resulting HIV-1 proviral clones are referred to as psHIV-wt, psHIV-Ile, psHIV-His, and psHIV-Phe, respectively (Fig. 1).

Plasmid pLGRNL used to express vesicular stomatitis virus G glycoprotein (VSV-G) has been previously described (2).

In Vitro Transcription

Plasmid p67YF0, which contains the yeast tRNA\text{Phe} gene, was kindly provided by Dr. Uhlenbeck (16). p67YF0 contains a T7 promoter directly adjacent to the yeast tRNA\text{Phe} gene and a \textit{BstNI} restriction site at the 3' end of the gene. Runoff transcription of the \textit{BstNI}-digested p67YF0 would give a 76-nucleotide RNA identical to an unmodified
yeast tRNA\textsuperscript{phe}. The transcription reactions were carried out using a commercial in vitro transcription kit (GIBCO), following the instruction of manufacturer.

**Transfection and Selection of Drug-Resistant Cell Lines**

The human embryonal kidney cell line 293T was used for all transfections. Calcium-phosphate (Qiagen) was used to transflect plasmid DNA to produce virus as instructed by the manufacturer. When cotransfecting both plasmid DNA and tRNA, lipofectin (GIBCO) was used following manufacturer's instruction. At 48 h posttransfection, the supernatants were collected and filtered through a 0.45-\(\mu\)m pore-size filter (Nalgene). Virus production was monitored by measuring the p24 antigen levels using a commercial ELISA kit (Coulter Laboratories).

Different dilutions of viruses produced by cotransfections were used to infect HeLa H1 cells. At 2 h after infection, the cells were washed once and then fresh DMEM plus 10\% fetal calf serum and 1\% antibiotics was added. At 24 h after infection, the medium was replaced with the selection medium (DMEM containing 10\% fetal calf serum, 1\% antibiotics, 20 mM HEPES [pH 7.5], 250 \(\mu\)g of xanthine per ml and 50 \(\mu\)g mycophenolic acid per ml). This medium was changed every 2 to 3 days until colonies of drug-resistant cells were formed (10 to 14 days). To determine the colony number, colonies were fixed by 5\% trichloroacetic acid (TCA) solution and stained with 2\% Coomassie blue. For sequence analysis, individual colonies were isolated using cloning cylinder (Specialty Media) following the instructions of manufacturer. Each isolated colony was expanded in selection medium and used to isolate chromosomal DNA by using a Wizard DNA isolation kit (Promega). The U5-PBS region of integrated HIV-1 provirus
RESULTS

Construction of HIV-1 Proviral Genomes with a PBS Complementary to tRNA^His or tRNA^Ile

In a previous study, we described the construction and characterization of complete HIV-1 proviral genomes in which the PBS was changed to be complementary to tRNA^His or tRNA^Ile (22). Characterization of these viruses revealed the propensity to revert back to a PBS complementary to tRNA^Lys following limited in vitro culture. To ascertain whether the cognate tRNA was actually used for initiation of reverse transcription, we generated modified genomes in which only the first nine nucleotides of PBS were complementary to tRNA^His or tRNA^Ile. Analysis of the PBS regions from cultures obtained early after virus growth again revealed the presence of complete PBS complementary to the cognate tRNA (22). Based on these results, we concluded that the cognate tRNA was used for the initiation of reverse transcription. A hallmark of all the analysis of the U5 regions of the virus that used alternative tRNA though was the presence of numerous nucleotide changes (5, 22). Since these viruses had undergone multiple rounds of infection, it was not clear whether the changes in U5 were absolutely essential for the virus to use the alternative tRNA to initiate reverse transcription. To
address this issue, we constructed defective HIV-1 proviral genomes that contained PBS complementary to tRNA\textsuperscript{His} or tRNA\textsuperscript{Ile}. The envelope gene of these genomes was replaced with the gene \textit{gpt} (Fig. 1). Cotransfection of these defective proviral genomes with a gene encoding VSV-G resulted in the production of pseudoviruses which have the capacity to undergo a single round of infection; the infected cells become resistant to mycophenolic acid following successful integration of the provirus.

We first determined if the inclusion of the PBS complementary to tRNA\textsuperscript{His} or tRNA\textsuperscript{Ile} would affect the production of virus in the complementation assay. For these studies, plasmids were co-transfected into 293T cells and the supernatants were analyzed for production of virus particles by p24 antigen assay (Fig. 2). Our analysis revealed that similar levels of p24 antigen were produced from cells co-transfected with the proviral genomes containing either the wild-type PBS or PBS complementary to tRNA\textsuperscript{His} or tRNA\textsuperscript{Ile}. Further experiments were performed by varying the ratio of plasmids encoding the VSV-G envelope protein and the HIV-1 proviral genome to optimize the production of virus particles as measured by p24 (data not shown).

Analysis of the PBS in Cell Clones Obtained After Infection of HIV-1 With a PBS Complementary to tRNA\textsuperscript{His} or tRNA\textsuperscript{Ile}

To determine the effect of the PBS alternations, we next measured the numbers of drug-resistant cell colonies obtained after infection with the wild-type virus or viruses that contained a PBS complementary to tRNA\textsuperscript{His} or tRNA\textsuperscript{Ile}. Different amounts of viruses were used to establish the linear range for the assay. Extrapolation from the linear region revealed that under our experimental conditions, \textasciitilde{} 160 drug-resistant colonies per 100
FIG. 1. Defective HIV-1 proviral genomes. (A) The defective HIV-1 proviral genome is depicted. The gpt gene under control of the SV40 early promoter has been substituted for the env gene. To generate pseudotyped viruses, this plasmid was cotransfected with the plasmid encoding VSV-G protein. Infection of cells with the pseudotyped virus confers resistance to mycophenolic acid. (B) Substitution of the PBS to be complementary to tRNA^Ile^, tRNA^His^, or yeast tRNA^Phe^ The defective proviral genomes containing the mutant PBS were designated as psHIV-Ile, psHIV-His, or psHIV-Phe, respectively. The defective proviral genome with wild-type PBS is referred to as psHIV-wt.
FIG. 2. Release of viruses following transfection of defective proviruses. 293T cells were cotransfected with the designated proviruses and the VSV-G expression plasmid. At 48 h posttransfection, the supernatants were analyzed for released p24 antigen by ELISA. The mean values of p24 obtained from three independent transfections with standard deviations is depicted. The designations psHIV-wt, psHIV-Ile, and psHIV-His correspond to pseudoviruses with PBS complementary to tRNA$^{\text{Lys}}$, tRNA$^{\text{Ile}}$, or tRNA$^{\text{His}}$, respectively.
picograms of p24 were obtained using the wild-type virus. In contrast, we reproducibly obtained three- to fivefold less drug-resistant cell colonies with an equal amount of p24 from viruses that contained a PBS complementary to tRNA^{His} or tRNA^{Ile} (Fig. 3).

The DNA sequence of the integrated PBS from individual drug-resistant cell colonies were next determined. Colonies were grown and the high molecular weight DNA was isolated. Using PCR primers specific for the U5-PBS region, we amplified this region followed by subcloning and DNA sequence analysis (Table 1). Analysis of the colonies obtained from infection with wild-type virus revealed that all of the PBS were complementary to tRNA^{Lys,3}. From the cell clones derived from infection of virus with a PBS complementary to tRNA^{Ile}, the majority of the PBS recovered were complementary to tRNA^{Ile}, with the exception of a single PBS complementary to tRNA^{Lys,3}. Only PBS complementary to tRNA^{His} were recovered from colonies obtained following infection with

<table>
<thead>
<tr>
<th>PBS of input provirus</th>
<th>No. of cell clones used for sequencing</th>
<th>Sequence of integrated provirus PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS^{Lys,3} (wt)^b</td>
<td>12</td>
<td>12/12: PBS^{Lys,3}c</td>
</tr>
<tr>
<td>PBS^{Ile}</td>
<td>14</td>
<td>13/14: PBS^{Ile}</td>
</tr>
<tr>
<td>PBS^{His}</td>
<td>12</td>
<td>1/14: PBS^{Lys,3}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12/12: PBS^{His}</td>
</tr>
</tbody>
</table>

^a Envelope-deficient proviruses with PBS complementary to tRNA^{Lys,3} (wt), tRNA^{Ile}, or tRNA^{His} were co-transfected with plasmid encoding VSV-G. The resultant pseudoviruses were used to infect cells to induce drug-resistant colonies. Individual drug-resistant colonies were cloned and the PBS sequence of integrated proviruses determined.

^b Designated tRNA^{Lys,3}, tRNA^{Ile}, and PBS^{His} represent PBS complementary to tRNA^{Lys,3}, tRNA^{Ile}, or tRNA^{His}, respectively.

^c Number of independent PCR clones and number of designated sequence recovered.
FIG. 3. Comparison of infectivity of HIV-1 viruses with a PBS complementary to tRNA\textsuperscript{Ile} (psHIV-Ile) or tRNA\textsuperscript{His} (psHIV-His) with the wild type virus (psHIV-wt). The designated pseudoviruses generated by co-transfection of proviruses with VSV-G plasmid were measured by p24 ELISA. Serial dilutions of each virus (p24) were used to infect cells followed by selection in mycophenolic acid. The average numbers of drug-resistant colonies derived from independent virus infections is depicted.
the virus containing a PBS complementary to tRNA\textsuperscript{His}. Taken together, the results of these studies clearly demonstrate that in the initial round of infection virus with a PBS complementary to alternative tRNA utilizes that tRNA for initiation of reverse transcription. The use of tRNA\textsuperscript{Lys}\textsubscript{3} as primer by virus with a PBS complementary to tRNA\textsuperscript{Ile} in first-round of replication is consistent with our previous observation that following in vitro culture, virus containing a PBS complementary to tRNA\textsuperscript{Ile} reverted back to wild type faster than virus with a PBS complementary to tRNA\textsuperscript{His} (22).

Complementation of an HIV-1 Provirus With a PBS Complementary to Yeast tRNA\textsuperscript{Phe}

In previous studies, we had identified and characterized an HIV-1 provirus in which the PBS was genetically engineered to be complementary to the 3' terminal 18 nucleotides of yeast tRNA\textsuperscript{Phe}. This virus was not infectious in our tissue culture system unless we changed the first six nucleotides of the PBS to be complementary to tRNA\textsuperscript{Lys}\textsubscript{3}, then the virus used tRNA\textsuperscript{Lys}\textsubscript{3} to initiate reverse transcription (20, 21). To further characterize this virus, we engineered the PBS complementary to yeast tRNA\textsuperscript{Phe} into the defective proviral genome. Transfection of this defective proviral genome with the plasmid encoding the VSV-G generated similar levels of p24 antigen in the supernatant as the cotransfection of the proviral genome containing the wild-type PBS with the plasmid encoding VSV-G (data not shown). Upon infection of cells with the pseudotyped virus containing the PBS complementary to tRNA\textsuperscript{Phe}, we were unable to recover any substantial numbers of drug-resistant cell colonies. Thus consistent with our previous studies, the virus with a PBS complementary to yeast tRNA\textsuperscript{Phe} had greatly reduced infectivity. Since the results from
the previous section had shown that in the first-round infection, viruses predominantly utilized the tRNA complementary to the PBS, we wanted to determine whether the infectivity of virus with the PBS complementary to yeast tRNA\(^{\text{Phe}}\) could be restored by providing yeast tRNA\(^{\text{Phe}}\) in trans. For these studies, we utilized three different sources of yeast tRNA\(^{\text{Phe}}\): a commercially available preparation of isolated yeast tRNA\(^{\text{Phe}}\), a second preparation of isolated yeast total tRNAs, and a preparation of yeast tRNA\(^{\text{Phe}}\) obtained from in vitro transcription using a cDNA template. Previous studies have found the yeast tRNA\(^{\text{Phe}}\) generated from in vitro transcription using this cDNA is similar to the wild type with the exception of the posttranscriptional modifications of specific bases (16). Using optimized ratio of the plasmid encoding the HIV-1 provirus with the PBS complementary to tRNA\(^{\text{Phe}}\) and the plasmid encoding VSV-G, we added increasing amounts of the various tRNAs up to 4 \(\mu\)g into the cotransfection mixture. The resultant pseudoviruses were normalized for p24 and used to infect cells followed by mycophenolic acid selection. As expected, without the addition of yeast tRNA, few colonies were recovered (<1 colony per nanogram of p24). However, with the addition of the yeast tRNA, we found a dose-dependent increase in the number of drug-resistant colonies (Fig. 4). The greatest number of colonies was obtained using tRNA\(^{\text{Phe}}\) isolated from yeast. We found a similar number of colonies using yeast tRNA\(^{\text{Phe}}\) from in vitro transcription of the cDNA encoding yeast tRNA\(^{\text{Phe}}\) (300-fold over background). The number of colonies obtained using in vitro transcribed yeast tRNA\(^{\text{Phe}}\) was also greater than that obtained using a similar amount of total tRNAs (where tRNA\(^{\text{Phe}}\) is in lower amounts) isolated from yeast. Taken together, the results of these studies demonstrate that cotransfecting the cognate tRNA with the defective HIV-1 proviral genome resulted in the production of a virus, which can undergo
FIG. 4. Complementation of psHIV-Phe virus by cotransfected yeast tRNA^{Phe}. Values represent average numbers of drug-resistant colonies derived from the infection of viruses generated by cotransfecting psHIV-Phe provirus and VSV-G expression plasmid with indicated amounts of isolated yeast tRNA^{Phe}, in vitro transcribed yeast tRNA^{Phe}, or yeast total tRNAs. The number of colonies obtained without cotransfection of yeast tRNA^{Phe} was < 1 colony per nanogram of p24.
a productive infection as measured by the capacity to generate drug-resistant cell clones.

To further investigate the specificity of the complementation, we performed co-transfection experiments with tRNAs isolated from rabbit or calf liver using two different tRNAs concentrations (2 and 0.2 µg) within the linear range of the assay in the cotransfection (Fig. 5). The isolated or in vitro transcribed yeast tRNA\textsuperscript{Phe} again gave the greatest numbers of colonies, although the absolute number of colonies were reduced compared to that presented in Fig. 4. These differences might be due to variations between independent transfections. More importantly, only background numbers of colonies (< 1 colony per nanogram p24) were obtained following cotransfection with different concentrations of rabbit or calf tRNAs, confirming the specificity of the complementation by yeast tRNA\textsuperscript{Phe}. To further characterize the reverse transcription reaction with the yeast tRNA\textsuperscript{Phe} as primer, we analyzed the PBS of the integrated proviruses obtained from individual cell clones (Table 2). All of the cell clones analyzed

<table>
<thead>
<tr>
<th>tRNAs cotransfected with psHIV-Phe</th>
<th>No. of cell clones used for sequencing\textsuperscript{a}</th>
<th>Sequence of integrated provirus PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast total tRNAs</td>
<td>4</td>
<td>4/4</td>
</tr>
<tr>
<td>Yeast tRNA\textsuperscript{Phe}</td>
<td>4</td>
<td>4/4</td>
</tr>
<tr>
<td>Yeast tRNA\textsuperscript{Phe} (in vitro transcribed)</td>
<td>19</td>
<td>18/19; PBS\textsuperscript{Phe}, 1/19: sequence complementary to full-length tRNA\textsuperscript{Phe}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Plasmid containing psHIV-Phe provirus and plasmid encoding VSV-G were cotransfected with indicated tRNA species to generate pseudviruses. These viruses were used to infect cells which were then selected with mycophenolic acid. Individual cell colonies were isolated and the PBS was amplified by PCR and the DNA sequence determined. Numbers refer to independent cell clones sequenced and number obtained with designated DNA sequence.

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FIG. 5. Specificity of tRNA complementation. 2 µg (A) or 0.2 µg (B) of indicated tRNAs isolated from the designated sources were added in co-transfections to complement psHIV-Phe virus. Bar graph illustrates average numbers of drug-resistant colonies derived from the infection of psHIV-Phe pseudoviruses with various tRNAs. The asterisk corresponds to less than 1 colony/ng p24.
contained a PBS complementary to yeast tRNA\textsuperscript{Phe}. Most interestingly, among the cell clones derived from infection of virus complemented by in vitro transcribed yeast tRNA\textsuperscript{Phe}, we found a single clone out of 19 clones contained a sequence complementary to the full length (76-nucleotide) tRNA\textsuperscript{Phe}; the majority though (18 clones) contained the PBS complementary to the 3' terminal 18 nucleotides of yeast tRNA\textsuperscript{Phe}. Taken together, the results of these studies demonstrate that the unmodified tRNA can function as a primer for initiation of reverse transcription when supplied in trans.

**DISCUSSION**

In this report, we have investigated the mechanism of selection of the tRNA used for initiation of HIV-1 reverse transcription. Using a defective provirus that can only undergo a single round of replication, we demonstrated that HIV-1 with a PBS complementary to tRNA\textsuperscript{His} or tRNA\textsuperscript{Ile} predominantly utilized these tRNAs for the first round of replication. The efficiency of the initiation of reverse transcription, as determined by production of drug resistant cell colonies, was three- to fivefold lower for the viruses that utilized tRNA\textsuperscript{His} or tRNA\textsuperscript{Ile} as primer compared to the wild-type virus. A complementation system was developed to study the selection of the tRNA used for initiation of reverse transcription. A defective provirus with a PBS complementary to yeast tRNA\textsuperscript{Phe} was constructed that was only infectious upon cotransfection of native (modified) yeast tRNA\textsuperscript{Phe} or tRNA\textsuperscript{Phe} obtained from in vitro transcription of a cDNA template.

The details for the selection of the tRNA primer used for initiation of reverse transcription are currently unknown. Previous studies from this laboratory, as well as others, have established that alteration of the PBS to be complementary to tRNA\textsuperscript{Phe} other
than tRNA$^{\text{Lys,3}}$ resulted in viruses able to utilize these alternative tRNAs for the initiation of reverse transcription (3, 5, 7, 22). The viruses though rapidly reverted to utilize tRNA$^{\text{Lys,3}}$ for initiation of reverse transcription after limited in vitro culture. Due to the complexity of the in vitro culture system, it was not possible to determine if the viruses with a PBS complementary to the alternative tRNAs predominantly used the alternative tRNAs for the first round of replication. That is, it was possible that viruses with the PBS complementary to different tRNAs could not discriminate during the first round of replication between the cognate tRNA and tRNA$^{\text{Lys,3}}$. If the latter was the case, it might provide support for a role of viral proteins, other than the complementarity between tRNA and PBS, in selection of the tRNA used to initiate reverse transcription. The results of the current study clearly establish that complementarity between the PBS and an alternative tRNA is a major determinant for the selection and use of this tRNA in initiation of reverse transcription. Our data indicate that the vast majority of the initial reverse transcription events utilize the tRNA primer complementary to PBS. Upon analysis of clones derived from infection of the virus with PBS complementary to tRNA$^{\text{Ile}}$, we did find a single clone with the PBS complementary to tRNA$^{\text{Lys,3}}$. Even during the first round of infection then, reverse transcription was initiated with tRNA$^{\text{Lys,3}}$ on a PBS complementary to tRNA$^{\text{Ile}}$. This result correlates with our previous finding that reversion of virus with a PBS complementary to tRNA$^{\text{Ile}}$ occurred very rapidly in the in vitro culture (22). Why tRNA$^{\text{Lys,3}}$ can be more effectively positioned onto a PBS complementary to tRNA$^{\text{Ile}}$ but not tRNA$^{\text{His}}$ is not clear. It should be noted that the PBS complementary to tRNA$^{\text{Ile}}$ or tRNA$^{\text{His}}$ have 9- and 7-bp mismatches, respectively, with the 3' terminus of tRNA$^{\text{Lys,3}}$. Thus, our results that tRNA$^{\text{Lys,3}}$ can be positioned onto the PBS complementary to
tRNA^{Ile} are not fully explained by greater base pair complementarity in the PBS. Most probably, the entire U5-PBS RNA structure also contributes, although not as strongly as complementarity with the PBS, to the utilization of a specific tRNA for initiation of reverse transcription. Further experiments, using this single round system, will be required to elucidate the features of the U5-PBS involved in selection of the tRNA primer.

The results from our current study do provide a clue as to why viruses with a PBS complementary to an alternative tRNA are unstable and revert back to utilize tRNA^{Lys,3} for initiation of reverse transcription. Using these single round analysis, we found that viruses with a PBS complementary to tRNA^{His} or tRNA^{Ile} were generally three- to fivefold less efficient in replication, as measured by generation of drug-resistant colonies. While this might not seem significant, this difference, when amplified over multiple rounds of infection, would probably result in a considerable difference in the replication of the virus mutant compared to the wild type. Thus viruses that can utilize tRNA^{Lys,3} for initiation of reverse transcription might have a significant replication advantage following multiple rounds of re-infection and would, most probably, dominate over the mutant viruses in a continuous culture system. In previous studies, we have found viruses that can stably utilize alternative tRNAs for initiation of reverse transcription (6, 19, 25). Additional mutations within U5 of these viruses were found following extended in vitro culture. At the time, we speculated that these additional mutations along with the PBS complementary to the alternative tRNA were required for the virus to more efficiently utilize this tRNA for reverse transcription. In support of this hypothesis, preliminary experiments show that defective provirus with a U5-PBS optimized for use of tRNA^{His}
generated a greater number of drug-resistant colonies, than the provirus with only a PBS complementary to tRNA^{His} (Q. Yu and C. D. Morrow, unpublished data).

The fact that alteration of the PBS to be complementary to alternative tRNAs resulted in the use of these tRNAs as reverse transcription primer in the single-round infection assay system prompted us to determine whether we could supply in trans the tRNA to be used for initiation of reverse transcription. For these studies, we made use of a previously described HIV-1 provirus in which the PBS was mutated so as to be complementary to yeast tRNA^{Phe}; under our in vitro culture conditions this virus was noninfectious (21). We introduced the PBS complementary to yeast tRNA^{Phe} into the defective proviral genome. The pseudotyped virus that contained a PBS complementary to yeast tRNA^{Phe} did not produce significant quantities of drug-resistant cell colonies. To restore virus infectivity, we cotransfected the provirus with yeast tRNA^{Phe}; both native yeast tRNA^{Phe} (i.e., containing modified bases) as well as in vitro transcribed tRNA^{Phe} could complement the defective proviral genome. This is the first demonstration for the conditional replication of HIV-1 virus by providing the tRNA molecule in trans. The results of our studies are consistent with previous studies from Lund et al (8), who demonstrated that murine leukemia virus could be restored upon transfection of packaging cells with plasmids encoding a genetically modified tRNA. Our study differs from Lund et al (8) though, because we used in vitro transcribed tRNA for complementation. The results of our study lend support to the idea that the tRNA molecule interacts with the viral RNA genome within the cell (presumably in the cytoplasm) prior to release of virus, as opposed to the viral proteins selecting the tRNA used for initiation of reverse transcription. Interaction between a tRNA molecule and the viral RNA genome prior to
release of the virus would also explain the capacity of HIV-1 to utilize different tRNA molecules for the initiation of reverse transcription when the PBS is made to be complementary to these different tRNAs.

The establishment of a system whereby we can manipulate both the viral RNA genome as well as the tRNA primer by mutagenesis will also allow us to further define the regions of the tRNA molecules which are important for the interaction with the viral RNA genome. Although the modified bases found in tRNAs are not absolutely required for the tRNA to function as a primer for initiation of reverse transcription, the native yeast tRNA^{Phe} molecules did complement more effectively than the in vitro transcribed molecules, suggesting that the modified bases could facilitate the reverse transcription process. The modified bases could function by inhibiting the RT from copying more than 18 nucleotides from the 3' terminal region of tRNA primer during plus-strand synthesis (1). This idea is supported by our finding that one of the cell clones, which was derived from infection of the virus complemented by in vitro transcribed tRNA^{Phe}, contained the complete 76-nucleotide sequence of yeast tRNA^{Phe} in the PBS region of integrated virus. Presumably, this sequence was generated during the plus-strand synthesis when the RT utilized the tRNA^{Phe} as template for the generation of a plus strand PBS. Ultimately, the use of the in vitro transcribed tRNA^{Phe} in this system will help us to derive a detailed picture of which regions of the tRNA molecule are important for interaction with the viral RNA genome to function as a primer for initiation of reverse transcription. This information could be used in the future to design therapeutics targeted to disrupt the tRNA-PBS interaction, which is essential for the initiation of HIV reverse transcription.
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ESSENTIAL REGIONS OF THE tRNA PRIMER REQUIRED FOR HIV-1 INFECTIVITY

by

QIN YU AND CASEY D. MORROW

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ABSTRACT

Human immunodeficiency virus (HIV), like all retroviruses, requires a cellular tRNA as a primer for initiation of reverse transcription. In a previous study, we demonstrated that an HIV-1 with a primer binding site complementary to yeast tRNA<sup>Phe</sup> (psHIV-Phe) was not infectious unless yeast tRNA<sup>Phe</sup> was supplied in trans. This unique in vivo complementation system has now been used to define the elements of the tRNA required for HIV-1 replication. Mutant tRNA<sup>Phe</sup> with deletions in TΨC stem-loop, anticodon stem-loop, or D stem-loop of the tRNA were generated and assessed for the capacity to rescue psHIV-Phe. Mutant tRNA<sup>Phe</sup> with disrupted TΨC stem-loop did not rescue psHIV-Phe. In contrast, a mutant tRNA<sup>Phe</sup> without the D stem-loop was fully functional for the rescue. The tRNA anticodon stem-loop region was found to be important for efficient complementation. The results of our studies demonstrate for the first time the importance of specific structural and sequence elements of the tRNA primer for HIV-1 reverse transcription and define new targets for interruption of HIV-1 replication.

INTRODUCTION

Retroviruses use cellular tRNA as a primer to initiate reverse transcription (20). tRNA<sup>Lys</sup><sup>3</sup> is the natural primer for human immunodeficiency virus (HIV) and related lentiviruses. In HIV virions, the tRNA is bound to an 18-nucleotide region of the viral genome designated as the primer binding site (PBS), which is complementary to the 3'-terminal nucleotides of the tRNA. Studies from this laboratory, and others, have shown
that the complementarity between the PBS and the tRNA 3’ terminus is a major
determinant for the selection of a tRNA primer in HIV-1 (4, 11, 18, 30). Changing the
HIV-1 PBS to be complementary to tRNAs other than tRNA_{Lys}^3 resulted in viruses that
used the alternative tRNAs as primer in reverse transcription. However, these viruses
rapidly reverted to use tRNA_{Lys}^3 following in vitro culture unless an accompanying
region in the 5’ unique region (U5), the A-loop, was also mutated (10-13, 28). Even with
optimized A-loop-PBS combinations in the viral genome though, not all tRNAs were
stably used by viruses following in vitro culture (11), highlighting the complexity of the
interactions between HIV-1 genome and the tRNA primer.

Most in vivo studies on HIV-1 RNA-tRNA interactions have been focused on
understanding the sequence elements within the HIV-1 genome required for the selection
and use of tRNA primer. Although in vitro experiments exploring the interactions of
tRNA with HIV-1 reverse transcriptase (RT) or with viral RNA have been reported (2, 6-
8, 22), little is known about the sequence and structural requirements for a tRNA to be
selected and used in HIV-1 reverse transcription in vivo. Studies on the elements of the
tRNA primer important for yeast retrotransposon Ty1 reverse transcription have taken
advantage of techniques to genetically manipulate yeast cells (5, 14). A system has also
been described for murine leukemia virus which used genetically engineered tRNA to
complement a defective provirus (19). We recently established an in vivo
complementation system to allow identification of specific elements within the tRNA
that are important for the primer selection and use in HIV-1 reverse transcription (32).
The replication of a defective HIV-1 with a PBS complementary to yeast tRNA_{Phe}, which
previous studies have shown to be noninfectious (29), was restored when yeast tRNA\textsuperscript{Phe} was provided during virus production.

Yeast tRNA\textsuperscript{Phe} is an ideal candidate to examine the role of specific regions of tRNA structure in primer selection because its three-dimensional structure is well-characterized (23). The cloverleaf base-pairing yeast tRNA\textsuperscript{Phe} sequence folds into an L-shaped molecule, which is universal among tRNAs. An L-shaped tRNA can be divided into two main domains: the "top half", a minihelix motif, consists of the acceptor stem and T\textsuperscript{YC} stem-loop; and the "bottom half" includes the anticodon stem-loop, D stem-loop, and variable loop. In this study, a series of yeast tRNA\textsuperscript{Phe} mutants were made and tested for their capacity to restore psHIV-Phe virus infectivity. We found that, while the T\textsuperscript{YC} stem-loop and anticodon stem-loop of yeast tRNA\textsuperscript{Phe} are essential for complementation, the tRNA\textsuperscript{Phe} D stem-loop structure was not required. The results of these studies are discussed with respect to the elements of the tRNA required for primer selection and use and potential application in designing new therapeutics to disrupt this step in HIV-1 reverse transcription.

MATERIALS AND METHODS

Tissue Culture and Cell Lines

293T cells and HeLa H1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) with 10% fetal calf serum and 1% antibiotic-antimycotic
(penicillin/streptomycin/amphotericin cocktail; GIBCO) in a 37°C incubator supplied with 5% CO₂.

DNA Plasmids

The construction of plasmid psHIV-Phe was described in detail in our previous study (32). Briefly, psHIV-Phe contains a defective HIV-1 proviral genome in which the env gene was deleted and replaced by a drug-resistant gene gpt (xanthine-guanosine phosphoribosyl transferase) (Fig. 1). The PBS region of this provirus was mutated to be complementary to the 3'-terminal 18 nucleotides of yeast tRNA\textsuperscript{Phe}. Plasmid pLGRNL used to express vesicular stomatitis virus G glycoprotein (VSV-G) has been previously described (3).

In Vitro Transcription

Plasmid p67YF0, which contains the yeast tRNA\textsuperscript{Phe} gene, was kindly provided by Dr. O. C. Uhlenbeck (24). p67YF0 contains a T7 promoter directly adjacent to the yeast tRNA\textsuperscript{Phe} gene and a Bst\textit{NI} restriction site at the 3' end of the gene. "Run off" transcription of the Bst\textit{NI}-digested p67YF0 gives a 76-nucleotide RNA identical to the unmodified yeast tRNA\textsuperscript{Phe}. All transcription reactions were carried out using a commercial in vitro transcription kit (GIBCO), following the instructions of the manufacturer.

To generate tRNA\textsuperscript{Phe} mutants, cDNA fragments starting with sequence containing a T7 promoter (5'-CTGCAGTAATACGACTCATA) followed by the exact coding
sequence for one of the following yeast tRNA<sup>Phe</sup> mutants were made: the 35 mer, 45 mer, 46 mer, 47 mer, 55 mer, 62 mer, 62-loop, and 62-stem (see Figs. 2A, 3A, and 4A for the nucleotide sequences). These cDNA fragments were generated by annealing the plus-strand and the complementary minus-strand synthetic DNA oligomers at 94°C and then slowly cooling down to room temperature. Each of the cDNA fragments was used as a template to generate the encoded yeast tRNA<sup>Phe</sup> mutants by in vitro transcription.

**RNA Synthesis**

Synthetic RNA oligonucleotides 35 mer, 62 mer, 55 mer, 45 mer, 47 mer, 46 mer, and 62-UUU (Figs. 2A, 3A, and 5), generated using an Applied Biosystems 392 DNA/RNA synthesizer, were provided by Dr. S. Hajduk (University of Alabama at Birmingham). The synthetic oligonucleotides were gel purified prior to use in the complementation system. All RNAs were quantitated by UV absorption and by gel electrophoresis and ethidium bromide staining. The latter was followed by the use of Alpha-Imager (Alpha Innotech), which achieved quantitation through comparing the density of each RNA band with the standards.

**Transfection and Selection of Drug-Resistant Cell Lines**

The procedure used for complementation has been previously described (32, 33). Briefly, lipofectin (GIBCO) was used to cotransfect plasmid DNA (1 µg) and RNA oligonucleotides (2 µg) into 293T cells. At 36 h posttransfection, the supernatants of
transfected cells were collected and filtered through a 0.45-µm pore-size filter (Nalgene). Virus production was monitored by measuring the p24 antigen levels using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Coulter Laboratories). Different dilutions of viruses produced by cotransfections were used to infect HeLa H1 cells. At 2 h after infection, the cells were washed once and fresh DMEM with 10% fetal calf serum and 1% antibiotics was added. At 24 h after infection, the medium was replaced with the selection medium (DMEM containing 10% fetal calf serum, 1% antibiotics, 20 mM HEPES [pH 7.5], 250 µg/ml xanthine, and 50 µg/ml mycophenolic acid). This medium was changed every 2 to 3 days until colonies of drug-resistant cells were formed (10 to 12 days). Cell colonies were washed with phosphate buffered saline and stained with crystal violet solution (0.2% crystal violet, 25% isopropanol, and 5% acetic acid). For analysis of integrated proviral primer binding site, cell colonies were isolated and expanded in selection medium; chromosomal DNA was isolated using a Wizard genomic DNA purification kit (Promega). The U5-PBS region of integrated provirus in the isolated chromosomal DNA was amplified by PCR with the following primers: primer 1 (5'-TAGACCAGATCTGAGCCTGGGAGCTC-3'; nt 13 to 48), and primer 2 (5'-CTCCTTCTAGCCTCGCTGCTAGTC-3', complementary to nt 331 to 310). The PCR-amplified fragments were cloned into pGEM-T-Easy vector (Promega), and the resultant plasmid DNAs were amplified and prepared for sequence analysis.
Biotinylation or Radiolabeling of tRNAs

The biotin RNA labeling mix (Boehringer Mannheim) was used in the in vitro transcription as substrates to generate biotin-labeled (biotinylated) RNA oligonucleotides, following the instructions of the manufacturer. $^{35}$S-labeled tRNAs were generated using the MEGAscript transcription kit (Ambion) as instructed by the manufacturer, with the exception that the final concentration of uridine triphosphate (UTP) was changed from 7.5 mM to 0.75 mM (1/10 of other nucleotide triphosphates [NTPs]), and 10 mCi/ml $^{35}$S-UTP stock (ICN) was also added to achieve a final concentration of 1 mCi/ml in the reaction. The transcripts were purified by phenol/chloroform extraction and ethanol precipitation.

two microgram of biotin-labeled or $^{35}$S-labeled tRNAs was cotransfected into 293T cells with psHIV-Phe provirus and plasmid pLGRNL. At specified time points the transfected cells were washed with phosphate buffered saline, incubated with RNase A (10 µg/ml in phosphate buffered saline solution) for 20 min, and then washed again three times with phosphate buffered saline. Subsequently, total intracellular RNAs of the cells transfected with biotin-labeled tRNAs were isolated using TRI REAGENT (Molecular Research Center, Inc.). One percent of the total RNAs were spotted on positively-charged Nylon membrane (Boehringer Mannheim) followed by the use of a biotin luminescent detection kit (Boehringer Mannheim). The biotin-labeled tRNA was detected with strepavidin coupled to alkaline phosphatase (AP) and was visualized by adding the chemiluminescent AP-substrate CSPD. The cells transfected with $^{35}$S-labeled tRNAs were harvested by trypsinization and lysed in 10% trichloroacetic acid solution. Then
the precipitated nucleic acids were collected on a 0.45 μm membrane filter (Gelman Science). The dried filters were placed in scintillation fluor (Research Product International), and radioactivity was determined by counting.

RESULTS

In a previous study, we described a defective HIV-1 provirus with a PBS complementary to the 3'‐terminal 18 nucleotides of yeast tRNA_{phe}, designated as psHIV-Phe, which contains a drug-resistant gpt in place of the deleted env gene (Fig. 1) (32). Pseudoviruses derived from cotransfection of psHIV-Phe provirus and the plasmid encoding VSV-G protein were noninfectious as measured by the ability to induce resistance to mycophenolic acid. Yeast tRNA_{phe} added into the cotransfection restored the infectivity of the pseudovirus in a dose-dependent fashion.

Dissection of the tRNA Required for Complementation of psHIV-Phe

Since little is known about the region of the tRNA required for in vivo primer selection and use in reverse transcription, our strategy was to first generate deletion mutants of tRNA_{phe}. We first tested two tRNA_{phe} deletion mutants, a 35 mer and 62 mer (Fig. 2A). The 35 mer is the minihelix of yeast tRNA_{phe}, consisting of the acceptor stem and TYC stem-loop. The 62 mer represents a yeast tRNA_{phe} without the D stem-loop. Unless otherwise specified, yeast tRNA_{phe} mutants were generated by direct RNA synthesis, followed by cotransfection with psHIV-Phe provirus and the plasmid encoding VSV-G protein (pLGRNL); the resultant pseudoviruses were normalized for p24 and
FIG. 1. psHIV-Phe proviral genome. The defective psHIV-Phe proviral genome with a PBS complementary to yeast tRNA$^{Phe}$ is depicted. The gene encoding xanthine-guanosine phosphoribosyl transferase (gpt) under the control of SV40 early promoter was substituted for the env gene of HIV. The 18-nucleotide sequence of the PBS complementary to yeast tRNA$^{Phe}$ is referred to as PBS$^{Phe}$. To generate pseudoviruses, this plasmid was cotransfected with the plasmid encoding VSV-G protein either with or without tRNA. Successful infection of cells with the pseudoviruses confers resistance to mycophenolic acid.
FIG. 2. The capacity of the yeast tRNA<sub>Phe</sub> deletion mutants to restore psHIV-Phe infectivity. (A) The RNA nucleotide sequences of yeast tRNA<sub>Phe</sub> and mutant 35 mer and 62 mer. The 3'-terminal 18 nucleotides complementary to the PBS of psHIV-Phe are in bold. (B) Average numbers of drug-resistant colonies derived from the infection of psHIV-Phe pseudoviruses generated by cotransfecting psHIV-Phe provirus and VSV-G expression plasmid (pLGRNL) along with wild-type yeast tRNA<sub>Phe</sub>, 35 mer, or 62 mer. psHIV-Phe virus without tRNA complementation served as a negative control. (C) The stability of biotin-labeled tRNA<sub>Phe</sub> mutants following cotransfection. Biotin-labeled yeast tRNA<sub>Phe</sub> or mutants were cotransfected with psHIV-Phe provirus and pLGRNL into 293T cells. At 0 h or 36 h after cotransfection, total RNAs were isolated from the transfected cells, spotted on Nylon membrane, and visualized using a biotin luminescent detection kit. Biotin-labeled tRNA<sub>Phe</sub> was spotted directly on the membrane as a positive control (control 1). The cotransfection of unlabeled tRNA<sub>Phe</sub> with psHIV-Phe and pLGRNL served as a negative control (control 2); cells incubated with biotin-labeled tRNA<sub>Phe</sub>, psHIV-Phe, and pLGRNL in the absence of transfection reagent were also included as a control (control 3). The RNA stocks used for cotransfection were diluted and then spotted on the membrane as controls (labeled as input RNA). (D) Stability of <sup>35</sup>S-labeled tRNA<sub>Phe</sub> mutants following cotransfection. Similar experiments as described in panel C above were carried out with <sup>35</sup>S-labeled tRNAs in place of the biotin-labeled tRNAs. Nucleic acids were precipitated from the cells at 0 h, 18 h, or 36 h after cotransfections and collected to determine radioactivity by counting. The counts obtained at 0 h, 18 h, and 36 h were divided by the input amounts for each sample. The values presented represent percentages of the input amount.
used to infect HeLa cells, followed by selection for resistance to mycophenolic acid. A low number of drug-resistant colonies were recovered from the infection of psHIV-Phe virus complemented by 35 mer, which was not greater than the background levels of the assay (Fig. 2B). In contrast, the 62 mer restored the psHIV-Phe virus infectivity with a similar efficiency as the wild-type yeast tRNA\textsuperscript{Phe} (Fig. 2B). Similar results were found with a 62 mer generated from in vitro transcription, indicating that the ability of the RNA to function in the assay did not depend on the method of RNA generation (data not shown).

The complementation of psHIV-Phe requires the tRNA to present in the cells, be selected as primer through yet undefined mechanism, and be positioned at the PBS. To further investigate the differences between 35 and 62 mer with respect to complementation, the stability of the RNAs was analyzed following transfection. Biotin-labeled wild-type tRNA\textsuperscript{Phe}, 35 mer, and 62 mer were generated by in vitro transcription and cotransfected with psHIV-Phe and pLGRNL. The cotransfections were carried out by first incubating nucleic acids-lipofectin mixtures with the cells for 8 h. After the 8-h incubation, growth medium was added to the cells; we designated this as time point 0 h. At time point 0 or 36 h, the transfected cells were incubated with RNase A and washed extensively to eliminate any untransfected tRNA that might remain outside of the cells (Q. Yu and C. D. Morrow, unpublished data); total cellular RNAs were then isolated from the transfected cells and spotted on a membrane for the detection of biotin-labeled tRNA (Fig. 2C). Our analysis revealed that, after 8-h incubation with the cells (time point 0 h), less 35 mer was detected than wild-type tRNA\textsuperscript{Phe} or the 62 mer. The 62 mer
had a similar stability profile as the wild-type tRNA\textsuperscript{Phe}, at least up to 36 h after cotransfection, which corresponds to the time for harvesting pseudoviruses for infections (Fig. 2C). The results from this analysis suggested that the 35 mer might be less stable than the 62 mer following transfection. To further address this possibility, we repeated the above experiments with $^{35}$S-labeled tRNA\textsuperscript{Phe}, 35 mer, and 62 mer (Fig. 2D). All three RNAs were detected at 36 h posttransfection at levels 2-5% of the amounts used in transfection. However, at 0 h time point there was approximately twice as much 62 mer and wild-type tRNA\textsuperscript{Phe} as the 35 mer, again suggesting the 35 mer is less stable following transfection. Given the differences in stability between the 62 mer and 35 mer, we have not pursued the analysis of the 35 mer to complement psHIV-Phe. Instead, elements within the 62 mer were further analyzed to explore the role of different regions of the tRNA in complementation. A 47 mer containing partial deletion of the TΨC stem-loop was generated (Fig. 3A). The acceptor stem and anticodon stem-loop were predicted to remain intact in the 47 mer by the RNA secondary-structure modeling program M-fold (9, 21, 34). Although the 47 mer remained stable after cotransfection, the RNA had a very low capacity to rescue psHIV-Phe virus (Fig. 3B and C), indicating the importance of an intact TΨC stem-loop for complementation.

Role of the Anticodon Stem-Loop Region of tRNA in Primer Selection and Use in Reverse Transcription

To address the role of the anticodon stem-loop and variable loop of the tRNA in primer selection and use in HIV-1, three new tRNA\textsuperscript{Phe} mutants based on the 62 mer were
FIG. 3. Complementation of psHIV-Phe virus by cotransfected yeast tRNA\textsuperscript{Phe} mutants. (A) The sequence of four yeast tRNA\textsuperscript{Phe} mutants generated by further deletion within the 62 mer. The 3'-terminal 18 nucleotides that are complementary to the PBS of psHIV-Phe are in bold. (B) The numbers of drug-resistant colonies derived from infection of the complemented pseudoviruses; psHIV-Phe virus without complementation was used as a control. (C) The stability of the tRNA\textsuperscript{Phe} mutants following cotransfection. The biotin-labeled yeast tRNA\textsuperscript{Phe} or mutants cotransfected with psHIV-Phe and pLGRNL were recovered from the transfected cells and analyzed as described in Fig. 2C.
generated and tested in our complementation system: the 55 mer (the anticodon loop was deleted), 45 mer (both anticodon stem and loop were deleted), and 46 mer (the anticodon stem and variable loop were deleted) (Fig. 3A). Upon infection of the cells with the psHIV-Phe pseudoviruses complemented by each of these mutants, we recovered fewer drug-resistant colonies compared to the virus complemented by 62 mer, although the values obtained for 55 mer and 45 mer were always above background (Fig. 3B). The lower complementation capacity of these mutants was not due to reduced stability compared to the wild-type tRNA<sup>Phe</sup> (Fig. 3C). To confirm the use of yeast tRNA<sup>Phe</sup> mutants as primers for reverse transcription by the rescued psHIV-Phe viruses, the PBS sequences of integrated psHIV-Phe proviruses were analyzed. Chromosomal DNA was isolated from cell clones derived from the infection of psHIV-Phe virus complemented by the 62 mer, 55 mer, or 45 mer. The proviral U5-PBS region in chromosomal DNA was amplified by PCR, followed by subcloning and sequence analysis. The results showed that all the PBS sequences recovered were complementary to the 3'-terminal 18 nucleotides of yeast tRNA<sup>Phe</sup>. Since the PBS sequence of integrated HIV provirus reflects the actual primer used in reverse transcription, the yeast tRNA<sup>Phe</sup> mutants examined had functioned as reverse transcription primers in the rescued psHIV-Phe viruses (data not shown).

To further delineate the features of anticodon stem-loop structure for the selection and use of the tRNA in reverse transcription, two additional mutants were designed. In the mutant 62-loop, C<sub>14</sub>-G<sub>16</sub> to G<sub>14</sub>-C<sub>16</sub> change disrupts the anticodon stem structure; in the second mutant, designated as 62-stem, a C<sub>26</sub>-G<sub>28</sub> to G<sub>28</sub>-C<sub>28</sub> change in addition to the
C_{14}-G_{16} to G_{14}-C_{16} change was introduced to restore the complementarity between the two strands of the anticodon stem (Fig. 4A). The disruption of the anticodon stem-loop structure in the mutant 62-loop and the restoration of this structure in the mutant 62-stem were predicted by the M-fold program (9, 21, 34). These mutant RNAs were generated by in vitro transcription and analyzed for their capacity to complement psHIV-Phe virus. Surprisingly, both mutant 62-loop and 62-stem showed a similar capacity in rescuing psHIV-Phe virus as the 62 mer (Fig. 4B), and no difference was detected between the stability of the mutants and the wild-type tRNA^phe following cotransfection (Fig. 4C). Taken together, the results of our analysis established that the anticodon region is essential for primer selection and use in HIV-1 reverse transcription, whereas the native anticodon stem-loop structure per se is not required.

The anticodon of tRNA^{Lys,3} has been shown to interact with the A-loop in the U5 region of HIV-1 RNA (6). Although previous studies have shown that a certain combination of A-loop-PBS resulted in viruses that stably maintained a PBS complementary to an alternative tRNA (e.g. tRNA^{His} or tRNA^{Met}), other A-loop-PBS mutations were not stable (e.g. tRNA^{Phe}) (11). Based on the results of this and other studies, we believe that the U5-PBS interaction with tRNA is complex and, most probably, depends upon the formation of RNA structures in the U5. As an alternative to mutating the A-loop region of U5 to correspond to the anticodon of tRNA^{Phe}, we generated a tRNA^{Phe} mutant (62-UUU) in which the anticodon was altered to that of tRNA^{Lys,3} (GAA to UUU) (Fig. 5A). This mutant should take advantage of the wild-type A-loop structure of psHIV-Phe genome for initiation of reverse transcription.
FIG 4. Comparison of the tRNA^Phe^ mutants with different structures in the anticodon stem-loop region. (A) Two mutants were generated by altering nucleotides in the anticodon stem of mutant 62 mer. The C\textsubscript{14}-G\textsubscript{16} to G\textsubscript{14}-C\textsubscript{16} (outlined) change in the 62-loop was predicted to disrupt the anticodon stem. The 62-stem was generated by introducing mutations (C\textsubscript{26}-G\textsubscript{28} \rightarrow G\textsubscript{26}-C\textsubscript{28}) (outlined) into mutant 62-loop to restore the complementarity between the two strands. (B) Drug-resistant colonies derived from the infection of psHIV-Phe virus complemented by 62-loop or 62-stem; psHIV-Phe virus without tRNA complementation was used as a control. (C) Stability of the tRNA^Phe^ mutants following cotransfection. See Fig. 2C for description of the procedure.

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FIG. 5. Complementation of psHIV-Phe by a tRNA$^{\text{Phe}}$ mutant with the anticodon of tRNA$^{\text{Lys,3}}$. (A) The anticodon of tRNA$^{\text{Phe}}$ (GAA) was substituted with the anticodon of tRNA$^{\text{Lys,3}}$ (UUU, in bold) in the 62 mer (Fig. 2A) to generate mutant 62-UUU. (B) The numbers of drug-resistant colonies derived from the infection of psHIV-Phe virus rescued by 62-UUU, 62 mer, or tRNA$^{\text{Phe}}$. 

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Surprisingly, 62-UUU rescued the virus with a similar efficiency, as determined by colony numbers, as the 62 mer with a tRNA\textsuperscript{Phe} anticodon (GAA) (Fig. 5B). Thus, for the single-round infection analysis with tRNA\textsuperscript{Phe}, the complementarity with A-loop and PBS did not result in more efficient virus rescue. This result is consistent with other A-loop-PBS combinations (i.e., tRNA\textsuperscript{Met}) but is different from that found for tRNA\textsuperscript{His} (Q. Yu and C. D. Morrow, unpublished data).

**DISCUSSION**

Elements of the tRNA essential for primer selection and use in HIV-1 reverse transcription were delineated by using the yeast tRNA\textsuperscript{Phe} mutants to complement the replication of a defective HIV-1 virus, psHIV-Phe. Analysis of tRNA\textsuperscript{Phe} mutants revealed that, while the T\textsuperscript{Ψ}C stem-loop region was essential for complementation, the tRNA without the D stem-loop region was fully functional for complementation. Deletion of the anticodon stem and/or loop severely reduced the complementation capacity of the tRNA; however, mutations to disrupt or promote the anticodon stem-loop structure did not affect complementation (summarized in Fig. 6).

The results of our experiments provide the first insights into what tRNA sequence and structures are required for the selection and use of the tRNA primer for HIV-1 reverse transcription. Our experimental approach is unique because an in vivo, rather than an in vitro, system was used, which necessitates the tRNA to be selected from the intracellular milieu and positioned at the PBS in addition to being functional for initiation and completion of reverse transcription. In an in vitro system, reverse transcription can
FIG. 6. Summary of the effect of tRNA$^{\text{Phe}}$ mutations on primer selection and use in HIV-1 reverse transcription. The tertiary or "L-shape" representation of yeast tRNA$^{\text{Phe}}$ is depicted. Regions of the tRNA important for complementing the psHIV-Phe virus are marked with symbol ▲. The region not required for virus complementation is labeled with X. Circles indicate the positions of the point mutations affecting the secondary structure of the anticodon stem while not interfering with the virus complementation.
be accomplished with an RNA primer of 18 nucleotides, indicating that the complementarity between the PBS and a primer is sufficient for the use of the primer in reverse transcription (1). Our studies revealed that the requirement for primer selection and use in vivo is more complex than the simple complementarity between the potential primer and the PBS of HIV-1. We initially tested the minihelix of tRNA\textsuperscript{Phe} (35 mer) consisting of the acceptor stem and TΨC stem-loop. The tRNA minihelix motif has been proposed to be the oldest element of the tRNA (26) and was used by a primitive reverse Transcriptase (RT) encoded by the Mauriceville retroplasmid to initiate cDNA synthesis (15). The 35 mer though failed to rescue psHIV-Phe. A complication with the interpretation of this experiment was the difference in stability of 35 mer compared to the wild-type tRNA\textsuperscript{Phe} following cotransfection. However, tRNA\textsuperscript{Phe} mutants containing the minihelix and additional elements (45 mer, 46 mer, and 55 mer), which exhibited similar stability as tRNA\textsuperscript{Phe}, failed to rescue psHIV-Phe virus. Collectively, elements in the minihelix (35 mer) were probably not sufficient for the virus complementation. Our results do suggest that the minihelix contains essential elements for psHIV-Phe complementation. As demonstrated by the incapability of a tRNA\textsuperscript{Phe} mutant (47 mer) to rescue psHIV-Phe, one of these elements would be the TΨC stem-loop. Previous studies have suggested that the TΨC stem-loop of tRNA\textsuperscript{Lys,3} interacts with HIV-1 RT in vitro (22); the TΨC stem-loop was also proposed to interact with the U5 sequence of HIV-1 (20). These potential interactions may also occur between yeast tRNA\textsuperscript{Phe} and psHIV-
Phe, since the TΨC loop sequence is highly conserved among tRNAs. Further experiments would be required to address this issue.

A surprising result was that the mutant tRNA$^{\text{Phe}}$ without the D stem-loop functioned as effectively as wild-type tRNA$^{\text{Phe}}$ in complementing the psHIV-Phe virus. The D stem-loop is essential for tRNA tertiary folding through the D loop-TΨC loop interaction (16, 17). The lack of requirement for the D stem-loop for psHIV-Phe rescue strongly argues that the D stem-loop and, consequently, the tRNA tertiary structure are not essential for the tRNA primer selection and use in HIV-1 in vivo. This conclusion is consistent with the structure model of HIV-1 reverse transcription initiation complex proposed by Isel et al, which suggested that the D stem-loop of tRNA primer protrudes out of the initiation complex and does not interact with either viral RNA or reverse transcriptase (6, 8). Conversely, earlier studies suggested that the D stem-loop was important for HIV-1 RT-tRNA$^{\text{Lys,3}}$ binding in vitro (22, 25, 31). However, this interaction might be nonspecific, since HIV-1 RT binds with many other tRNAs with the same efficiency (27). It is possible the suggested tRNA-RT interaction, if it occurs in vivo, may not be involved in the specific selection and use of the tRNA primer for reverse transcription. Additional experiments will be required to address this possibility.

The deletion of the anticodon stem and/or loop severely impaired the capacity of the tRNA to rescue psHIV-Phe virus, while the disruption of the anticodon stem structure by point mutations did not affect the virus rescue. Alteration of the anticodon (from GAA to UUU) also did not compromise the ability of the tRNA to rescue. Collectively, our results may imply that the anticodon stem-loop region is important in
forming the correct three-dimensional structure of tRNA for primer selection, which was affected severely by the deletion of anticodon stem/loop and not by the point mutations. Alternatively, certain nucleotides within the anticodon stem-loop might be important for the viral genomic RNA-tRNA interaction. Brulé et al have suggested that the anticodon stem of tRNA<sup>Lys<sub>3</sub></sup> interacts with the U3 region during first-strand transfer of reverse transcription in vitro (2). The same region in psHIV-Phe U3 exhibits base-pairing potential with the tRNA<sup>Phe</sup> anticodon stem. Therefore, it is possible that the anticodon stem deletion mutant in our study was blocked in the strand-transfer step. Previous studies from this laboratory and others have suggested that the interaction between the anticodon of the tRNA primer and the A-loop region of HIV-1 RNA is important for primer selection (6, 12, 13, 28). However, the role of this interaction is not fully understood, since some but not all A-loop-PBS combinations resulted in viruses that stably used the alternative tRNAs. Furthermore, even when A-loop-PBS combinations resulted in viruses which stably use the alternative tRNAs (e.g., tRNA<sup>Met</sup> and tRNA<sup>His</sup>) as primers, the alternative A-loop may or may not enhance the virus infectivity. In a single-round infection assay, an A-loop region complementary to tRNA<sup>Met</sup> did not enhance the infectivity of the virus using tRNA<sup>Met</sup> as the primer, whereas an A-loop complementary to tRNA<sup>His</sup> did improve the infectivity of the virus using tRNA<sup>His</sup> as the primer (Q. Yu and C. D. Morrow. unpublished data). In this study, we addressed the question by generating a tRNA<sup>Phe</sup> mutant with the tRNA<sup>Lys<sub>3</sub></sup> anticodon to facilitate the anticodon and A-loop interaction. Surprisingly, the change of the tRNA<sup>Phe</sup> anticodon to match tRNA<sup>Lys<sub>3</sub></sup> did not result in enhanced virus-rescue (Fig. 5). One explanation for this
result is that the anticodon region of tRNA\textsuperscript{Phe} may interact with an alternative sequence in U5. This speculative interaction might play a similar role as the interaction between the tRNA\textsuperscript{Lys,3} anticodon and the A-loop, which could explain why the tRNA\textsuperscript{Phe} mutants with the anticodon of tRNA\textsuperscript{Lys,3} or tRNA\textsuperscript{Phe} are similar in rescuing psHIV-Phe. Alternatively, our single-round complementation system may not be sensitive enough to detect the contribution of the tRNA\textsuperscript{Lys,3} and A-loop interaction to the virus infectivity. Additional experiments will be required to address these possibilities.

In summary, the results of our studies provide the first characterization of the critical elements of the tRNA required for HIV-1 reverse transcription in vivo. Since a complete tRNA molecule was not required to complement the defective virus, it may be possible to use this information to design small RNAs that would compete with tRNA\textsuperscript{Lys,3} in primer selection and disrupt HIV reverse transcription without interfering with the natural cellular function of tRNA in protein synthesis. Further delineation of the important elements of the tRNA required for primer selection will aid in the understanding of the interaction between the tRNA and U5-PBS of the virus, which will facilitate the development of new therapeutics designed to inhibit HIV replication.

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ELEMENTS OF THE tRNA ACCEPTOR STEM AND TYC STEM-LOOP REQUIRED FOR HIV-1 INFECTIVITY

by

QIN YU AND CASEY D. MORROW

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ABSTRACT

In a previous study, we have reported the use of an in vivo complementation system to identify elements of a tRNA required for the primer selection and use in HIV-1 replication. In this system, a mutant human immunodeficiency virus type 1 (HIV-1) with a primer binding site (PBS) complementary to yeast tRNA\(^{\text{Phe}}\) (psHIV-Phe) relies on exogenous yeast tRNA\(^{\text{Phe}}\) as reverse transcription primer for infectivity. Here, we have used this system to investigate the role of nucleotides in tRNA acceptor stem and TΨC stem-loop in HIV-1 replication. The tRNA\(^{\text{Phe}}\) mutants which contain two (CA) or four base pair (ACCA) deletions at the 3' end retained the capacity to complement psHIV-Phe. Extension of the 5' nucleotides of tRNA\(^{\text{Phe}}\), with or without complementarity to the 3' ACCA sequence, resulted in tRNA\(^{\text{Phe}}\) with reduced capacity for complementation. However, extension of the 3' end of the tRNA with sequences complementary to the U5 region of the viral genome restored the capacity for complementation. Further analysis of 5' and 3' ends of tRNA\(^{\text{Phe}}\) suggested an importance for an intact acceptor stem RNA structure for complementation. Analysis of tRNA\(^{\text{Phe}}\) mutants with single-nucleotide changes in the TΨC stem-loop region that is complementary to the PBS revealed an importance for this region in complementation. Most surprisingly, additional mutations at nucleotides 54-56 in tRNA\(^{\text{Phe}}\), which are not part of the 18 nucleotides complementary to the PBS, were found to affect the capacity of the tRNA\(^{\text{Phe}}\) to rescue psHIV-Phe infectivity. The results of our studies are discussed with respect to the elements of the tRNA required for selection and use in HIV-1 replication.
INTRODUCTION

Retroviruses use cellular tRNAs as primers for initiation of reverse transcription (15). Human immunodeficiency virus (HIV) exclusively uses RNA^{Lys,3} as the primer for reverse transcription. tRNA^{Lys,3} is selected from the cellular tRNA pools, encapsidated into HIV virions, and positioned at the primer binding site (PBS), which is near the 5' end of the viral RNA genome. Subsequently, reverse transcription is initiated from the 3'-end –OH group of the PBS-bound tRNA^{Lys,3} (15).

The mechanism for tRNA^{Lys,3} primer selection and use in HIV replication is not understood. Previous studies have shown that tRNA-PBS interaction is a major determinant for the selection and use of a tRNA as reverse transcription primer in HIV-1 (7, 11, 14, 23). Substitution of the HIV-1 PBS with sequence complementary to alternative tRNAs resulted in viruses that used these alternative tRNAs as primers for reverse transcription. However, the HIV-1 constructed with the PBS complementary to alternative tRNAs reverted back to use tRNA^{Lys,3} after a short term of in vitro culture (7, 11, 14, 23). The results of these studies pointed to a more complex interaction between the tRNA and viral genome than just the 3' terminal nucleotides of the tRNA and the PBS. Additional interactions between HIV-1 RNA and tRNA^{Lys,3} have been identified from chemical and enzymatic analysis of tRNA−U5-PBS complexes (9, 10). One such interaction is between an RNA stem loop in U5 (A-loop) and the anticodon of tRNA^{Lys,3}. The importance of the A-loop in the selection of the tRNA was established by studies from this laboratory which have shown that some, but not all, A-loop-PBS combinations allow HIV-1 to stably use alternative tRNAs for initiation of reverse transcription (12, 13, 22). Recently, the anticodon stem sequence of tRNA^{Lys,3} was also proposed to interact...
with the U3 sequence of HIV-1 RNA genome to promote strand transfer in reverse transcription (2). The importance of this interaction in selection and use of tRNA\textsuperscript{Lys} has not been established using in vivo experiments.

Elucidation of determinants within tRNA\textsuperscript{Lys} for primer selection and use in HIV-1 is essential to understand the mechanism of reverse transcription. Inherently, these experiments are difficult because of the inability to modulate the levels of tRNA\textsuperscript{Lys} within the cell to assess the effects of tRNA\textsuperscript{Lys} mutations on HIV-infectivity. To circumvent this problem, we recently developed an in vivo complementation system to study the tRNA primer selection and use in HIV-1 (25). In this system, the infectivity of a defective HIV-1 (psHIV-Phe) depends upon an exogenous reverse transcription primer, yeast tRNA\textsuperscript{Phe}. We have used this system to identify elements of tRNA\textsuperscript{Phe} that are important for the primer selection and use in HIV-1. In our previous study, the anticodon stem-loop and TΨC stem-loop, but not the D stem-loop of tRNA\textsuperscript{Phe}, were found to be important for psHIV-Phe infectivity (26). In this work, we further investigated the role of tRNA\textsuperscript{Phe} acceptor stem and TΨC loop in the primer selection and use in HIV-1. The results demonstrate that, for the infectivity of psHIV-Phe, the single-stranded ACCA sequence at the tRNA 3' end was not essential; base pair complementarity between the 5' and 3' end of the tRNA is critical for virus infectivity. Nucleotide changes in the TΨC region resulted in tRNA\textsuperscript{Phe} mutants unable to complement psHIV-Phe. The role of the acceptor stem and the TΨC loop in primer selection and use in reverse transcription is discussed.
MATERIALS AND METHODS

Tissue Culture and Cell Lines

293T cells and HeLa H1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and 1% antibiotics in a 37°C incubator supplied with 5% CO₂.

DNA Plasmids

The construction of plasmid psHIV-Phe was described in detail in our previous study (25). Briefly, psHIV-Phe contains a defective HIV-1 proviral genome in which the env gene was deleted and replaced by a drug-resistant gene gpt (xanthine-guanosine phosphoribosyl transferase) (Fig. 1). The PBS region of this provirus was mutated to be complementary to the 3'-terminal 18 nucleotides of yeast tRNA^Phe. Plasmid pLGRNL used to express vesicular stomatitis virus G glycoprotein (VSV-G) has been previously described (3).

In Vitro Transcription

Plasmid p67YF0, which contains the yeast tRNA^Phe gene, was kindly provided by Dr. O. C. Uhlenbeck (21). p67YF0 contains a T7 promoter directly adjacent to the yeast tRNA^Phe gene and a BstNI restriction site at the 3' end of the gene. "Run off" transcription of the BstNI-digested p67YF0 gives a 76-nucleotide RNA identical to the unmodified yeast tRNA^Phe. All transcription reactions were carried out using the MEGASHortscript transcription kit (Ambion), following the instructions of the manufacturer.
To generate tRNA$^{\text{Phe}}$ mutants, cDNA fragments starting with sequence containing a T7 promoter (5'-CTGCAGTAATACGACTCACTATA) followed by the exact coding sequence for one of the following yeast tRNA$^{\text{Phe}}$ mutants were made: tRNA-GGU, tRNA-GGU-4, tRNA-GGU-9, tRNA-ACCA (Fig. 2B); tRNA$\Delta$AC, tRNA$\Delta$ACCA (Fig. 1A); tRNA$\Delta$70-76, tRNA$\Delta$GCG$\Delta$ACCA, tRNA$\Delta$GCG, tRNA-A$_5$C$_4$, tRNAU$_2$A$_4$ (Fig. 3A); tRNA-G$_{59}$G$_{61}$, tRNA-G$_{59}$, tRNA-G$_{61}$ (Fig. 4A); tRNA-A$_{54}$AG, tRNA-U$_{56}$, and tRNA-A$_{56}$ (Fig. 5A) (See the figures for the nucleotide sequences). These cDNA fragments were generated through PCRs using overlapping plus-strand and minus-strand synthetic DNA oligomers. Each of the resultant cDNA fragments was used as a template to generate the encoded yeast tRNA$^{\text{Phe}}$ mutants by in vitro transcription. The yeast tRNA$^{\text{Phe}}$ mutants were gel-purified prior to use in the complementation system. All RNAs were quantitated by UV absorption and by gel electrophoresis and ethidium bromide staining. The latter was followed by the use of Alpha-Imager (Alpha Innotech), which achieved quantitation through comparing the density of each RNA band with known standards.

Transfection and Selection of Drug-Resistant Cell Lines

The procedure used for complementation has been previously described (25, 27). Briefly, lipofectin (GIBCO) was used to cotransfect the plasmid DNA and RNA oligonucleotides into 293T cells. At 36 h posttransfection, the supernatants of transfected cells were collected and filtered through a 0.45-µm pore-size filter (Nalgene). Virus production was monitored through the p24 antigen levels measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Coulter Laboratories). Different dilutions of viruses produced by cotransfections were used to infect HeLa H1 cells. At 2
h after infection, the cells were washed once and fresh DMEM with 10% FCS and 1% antibiotics was added. At 24 h after infection, the medium was replaced with the selection medium (DMEM containing 10% fetal calf serum, 1% antibiotics, 20 mM HEPES [pH 7.5], 250 μg/ml xanthine, and 50 μg/ml mycophenolic acid). This medium was changed every 2 to 3 days until colonies of drug-resistant cells were formed (10 to 12 days). The cell colonies were washed with phosphate buffered saline, stained with crystal violet solution (0.2% crystal violet, 25% isopropanol, and 5% acetic acid), and counted.

Biotinylation or Radiolabeling of tRNAs

The biotin RNA labeling mix (Boehringer Mannheim) or 35S-uridine triphosphate (UTP):nucleotide triphosphates (NTPs) mix (ICN) was used in the in vitro transcription as substrates to generate biotin-labeled or 35S-labeled RNA oligonucleotides, respectively. The transcripts were purified by phenol/chloroform extraction and ethanol precipitation.

Approximately 2 μg of biotin-labeled or 35S-labeled tRNAs were transfected into 293T cells. At specified time points the transfected cells were washed with phosphate buffered saline, incubated with RNase cocktail (40 U/ml in phosphate buffered saline solution, Ambion) for 15 min, and then washed again three times with phosphate buffered saline. Subsequently, total intracellular RNAs of the cells transfected with biotin-labeled tRNAs were isolated using TRI REAGENT (Molecular Research Center, Inc.). One percent of the total RNAs were spotted on positively-charged Nylon membrane (Boehringer Mannheim) for biotin detection. The biotin-labeled tRNA was detected with streptavidin coupled to alkaline phosphatase (AP) and was visualized by
adding the chemiluminescent AP-substrate, CSPD, which were carried out using a biotin luminescent detection kit (Boehringer Mannheim).

The cells transfected with $^{35}$S-labeled tRNAs were harvested by trypsinization and lysed in 10% trichloroacetic acid solution. Then the precipitated nucleic acids were collected on a 0.45 μm membrane filter (Gelman Science). The dried filters were placed in scintillation fluor (Research Product International), and radioactivity was determined.

RESULTS

To investigate tRNA primer selection and use in HIV-1 replication, we have established an in vivo complementation system that allows manipulation of the tRNA primer for HIV-1 reverse transcription (25). In this system, the infectivity of an HIV-1 with a PBS complementary to yeast tRNA$^{\text{Phe}}$ (psHIV-Phe) relies on exogenous yeast tRNA$^{\text{Phe}}$ as the primer. psHIV-Phe contains a drug-resistant gene, $gpt$, in place of the deleted env gene (Fig. 1A). Cotransfection of yeast tRNA$^{\text{Phe}}$, psHIV-Phe provirus, and a plasmid (pLGRNL) encoding VSV-G produces a pseudovirus that can undergo a single round of infection. Successful infection leads to the formation of mycophenolic acid-resistant cells. Therefore, the capacity of a tRNA$^{\text{Phe}}$ mutant to rescue psHIV-Phe can be determined from the number of drug-resistant colonies obtained following infection.

For a tRNA$^{\text{Phe}}$ mutant to rescue the virus, the tRNA needs to be selected from the cellular tRNA pool, encapsidated into virions, positioned at the PBS region, and used in reverse transcription to generate full-length viral DNA. All the tRNA$^{\text{Phe}}$ mutants in this study were generated by in vitro transcription using cDNA templates. After gel purification, the tRNA$^{\text{Phe}}$ mutants were cotransfected with psHIV-Phe provirus and
FIG. 1. The effect of tRNA\textsuperscript{Phe} 3'-end nucleotide deletions on complementation of psHIV-Phe. (A) psHIV-Phe proviral genome. The defective psHIV-Phe proviral genome contains a PBS complementary to yeast tRNA\textsuperscript{Phe} (PBS\textsuperscript{Phe}). The \textit{env} gene of HIV was substituted by the xanthine-guanosine phosphoribosyl transferase (\textit{gpt}) gene, under the control of SV40 early promoter. To generate pseudoviruses, this plasmid was cotransfected with the plasmid encoding VSV-G protein either with or without tRNA. Successful infection of cells with the pseudoviruses confers resistance to mycophenolic acid. (B) Illustrations of the positions of the nucleotides deleted in mutants tRNA\textsubscript{ACA} and tRNA\textsubscript{AACCA}. (C) The numbers of drug-resistant colonies derived from infection of the psHIV-Phe pseudoviruses complemented by mutants tRNA\textsubscript{ACA} and tRNA\textsubscript{AACCA}. 

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pLGRNL to generate pseudoviruses. The amount of the pseudoviruses generated was measured by a p24 ELISA and normalized for each infection. The intracellular stability of each mutant was analyzed using biotin-labeled or radiolabeled tRNA for transfection (26). All tRNA mutants shared similar stability profiles as tRNA\textsuperscript{Phe} up to 36 h after transfection under our experimental conditions (data not shown).

The 3’-End ACCA Sequence of tRNA\textsuperscript{Phe} Is Not Required for Complementation

The tRNA primer for HIV-1 reverse transcription binds with the viral PBS region through its 3’-end 18 nucleotides which encompass the tRNA acceptor stem and T\textsuperscript{YC} stem-loop (Fig. 1B). The last four nucleotides of the 3’ end are single stranded in tRNA\textsuperscript{Phe}, as in most other eukaryotic tRNAs. In vitro binding assays under physiological conditions indicated that the binding of oligonucleotides complementary to the tRNA 3’ sequence occurs through initial interaction with the 3’-end single-stranded nucleotides of the tRNA, followed by invasion in the acceptor stem and strand displacement (17, 18). The 3’-end unpaired CCA sequence of tRNA-like structure has also been shown to be required for initiation of reverse transcription by a primitive reverse transcriptase (RT) (5, 6). The results of these studies prompted us to test whether 3’ single-stranded nucleotides of the tRNA are important for the complementation of psHIV-Phe. To address the requirement for the 3’-end ACCA sequence of tRNA\textsuperscript{Phe}, we designed two deletion mutants in which two (tRNA\textsuperscript{AAC}) or four (tRNA\textsuperscript{AACCA}) nucleotides were removed from the tRNA\textsuperscript{Phe} 3’ end (Fig. 1B). Mutant tRNA\textsuperscript{AAC} rescued psHIV-Phe very effectively. Mutant tRNA\textsuperscript{AACCA} rescued the virus, although with a slightly lower efficiency compared to the wild-type tRNA\textsuperscript{Phe} (Fig. 1C). Thus, the presence of the
ACCA sequence on the 3’ end of tRNA\textsuperscript{Phe} was not absolutely required for rescue of the psHIV-Phe.

To further explore the affects of the acceptor stem alternation on the capacity of the tRNA\textsuperscript{Phe} to rescue virus, four tRNA\textsuperscript{Phe} mutants were generated and examined for their capacity to rescue psHIV-Phe virus (Fig. 2A). One mutant (tRNA-GGU) contained three additional nucleotides (GGU) predicted to base pair with the tRNA\textsuperscript{Phe} 3’ end single-stranded region. This mutant showed decreased capacity for complementation compared to the wild-type tRNA\textsuperscript{Phe} (Fig. 2A and B). To determine whether free 3’ nucleotides were required for the tRNA to rescue the virus, nucleotides were added to tRNA-GGU to generate a 3’ unpaired region. The resultant mutants tRNA-GGU+4 and tRNA-GGU+9 contained additional four (CUUC) and nine (CUUCUAGAG) nucleotides, respectively, at the 3’ end when compared to tRNA-GGU (Fig. 2A). The added nucleotides in both mutants were complementary to psHIV-Phe RNA sequence immediately upstream of the PBS. Interestingly, tRNA-GGU+4 restored psHIV-Phe infectivity with a similar efficiency as wild-type tRNA\textsuperscript{Phe}, whereas tRNA-GGU+9 showed a low virus-rescue capacity similar to that of tRNA-GGU (Fig. 2B). Finally, a mutant tRNA\textsuperscript{Phe} was generated which contained an ACCA instead of GGU sequence added to the 5’ end. This would presumably allow the tRNA 3’ ACCA sequence to remain unpaired (Fig. 2A). Surprisingly, tRNA-ACCA showed even lower virus-rescue capacity compared to tRNA-GGU (Fig. 2B).
FIG. 2. The psHIV-Phe rescue capacity of tRNA\textsuperscript{Phe} mutants with altered termini. (A) Illustrations of tRNA\textsuperscript{Phe} mutants with additional nucleotides at the 3' or 5' ends. The added nucleotides are in bold. Only the terminal portion of each tRNA\textsuperscript{Phe} mutant is illustrated. (B) The numbers of drug-resistant colonies derived from infection of the psHIV-Phe pseudoviruses complemented by the tRNA\textsuperscript{Phe} mutants.
Importance of Nucleotides in the Acceptor Stem for Rescue of Virus

Since the tRNA<sup>phe</sup> mutant without 3' ACCA sequence could rescue psHIV-Phe, we asked how many additional nucleotides could be removed from the tRNA 3' region without compromising virus-rescue capability. Seven nucleotides (position 70-76) at the 3' end of the tRNA were deleted in mutant tRNAΔ70-76 (Fig. 3A), and as a result, tRNAΔ70-76 contains an additional three-nucleotide deletion when compared to mutant tRNAΔACCA (Fig. 1B). Unlike tRNAΔACCA, tRNAΔ70-76 failed to rescue psHIV-Phe (Fig. 3B). These results suggest that the decreased complementarity between the tRNAΔ70-76 and the viral PBS might not be sufficient to support the tRNA-PBS annealing and/or initiation of reverse transcription. Alternatively, the reduced base-pairing in the acceptor stem of tRNAΔ70-76 might have precluded the tRNA from being selected as a primer. To address these possibilities, three 5'-end nucleotides (G<sub>1</sub>C<sub>2</sub>G<sub>3</sub>) were deleted from the previously described mutant tRNAΔACCA (Fig. 3A). The resultant mutant, tRNAΔGCGΔACCA, would have the same complementarity with the PBS as in tRNAΔACCA, whereas the same number of base pairs in the acceptor stem as in tRNAΔ70-76. Mutant tRNAΔGCGΔACGC could not rescue psHIV-Phe (Fig. 3B). Furthermore, mutant tRNAΔGCG with the 5' G<sub>1</sub>C<sub>2</sub>G<sub>3</sub> deletion and a wild type 3' end also failed to rescue the virus (Fig. 3A&B). These results pointed to the possibility that the nucleotide length of the base-paired acceptor stem was important for psHIV-Phe rescue. To address this point, mutants tRNA-<i>A</i><sub>2</sub>C<sub>4</sub> and tRNA-<i>U</i><sub>2</sub>A<sub>4</sub> were designed (Fig. 3A). With nucleotide substitutions at the same positions, tRNA-<i>A</i><sub>2</sub>C<sub>4</sub> was predicted to have reduced base-pairing in the acceptor stem, whereas tRNA-<i>U</i><sub>2</sub>A<sub>4</sub> was predicted to maintain all the base pairs in the acceptor stem. Remarkably, tRNA-<i>A</i><sub>2</sub>C<sub>4</sub> completely
FIG. 3. The role of the tRNA<sub>Phe</sub> acceptor stem structure in complementation of psHIV-Phe. (A) Illustrations of five tRNA<sub>Phe</sub> mutants with mutations at the acceptor stem. Only the terminal portion of each tRNA<sub>Phe</sub> mutant is illustrated. (B) The numbers of drug-resistant colonies derived from infection of the psHIV-Phe pseudoviruses complemented by the tRNA<sub>Phe</sub> mutants.
lost the capacity to rescue psHIV-Phe (Fig. 3B). The tRNA-U2A4 did rescue virus, although not as effectively as wild type tRNA\textsuperscript{Phe}. Taken together, these results indicated that the complementary base pairing in the tRNA acceptor stem, facilitated by three GC base pairs, was important for rescue of psHIV-Phe.

**Elements in the TΨC Stem-Loop Are Essential for psHIV-Phe Complementation**

To explore the importance of the nucleotides in the tRNA which are complementary to the 3' end of the PBS, mutations were introduced in the PBS-binding sequence located at the TΨC stem-loop (Fig. 4A). Surprisingly, one mutant, tRNA-G\textsubscript{59}G\textsubscript{61}, failed to rescue psHIV-Phe, despite the remaining 15-nucleotide complementarity between this mutant and the PBS (Fig. 4B). Further dissection of the G\textsubscript{59}G\textsubscript{61} mutation indicated that the mutation G\textsubscript{61} resulted in the mutant unable to rescue psHIV-Phe (Fig. 4B). To investigate the importance of nucleotides within the TΨC loop for rescue of psHIV-Phe, the highly conserved TΨC sequence was substituted with AAG in mutant tRNA-A\textsubscript{54}AG (Fig. 5A). Analysis of this tRNA mutant revealed an impaired capacity in rescuing psHIV-Phe (Fig. 5B).

Examination of the psHIV-Phe RNA sequence immediately downstream of the PBS revealed complementarity (5'-201UCGAA\textsubscript{205}-3') with the tRNA\textsuperscript{Phe} TΨC loop sequence (5'-5\textsubscript{4}TΨCGA\textsubscript{58}-3'). RNA modeling of this region predicted a nonbase-paired loop structure 3' to the PBS (Fig. 6A). Therefore, the inability of tRNA-A\textsubscript{54}AG to rescue the virus could be explained by disruption of potential interaction between the TΨC loop and the PBS-downstream sequence of HIV-1. To further address this possibility, we introduced a single mutation (C\textsubscript{56}→A\textsubscript{56}) in the TΨC loop, which would not favor the
FIG. 4. Mutations in the tRNA$^{\text{Phe}}$ T$^\Psi$C stem-loop affected complementation of psIIV-Phe. (A) Illustrations of the positions of the nucleotides mutated in mutants tRNA-$G_{59}G_{61}$, tRNA-$G_{59}$, and tRNA-$G_{61}$. (B) The psIIV-Phe rescue capacity of the tRNA$^{\text{Phe}}$ mutants as measured by drug-resistant colony numbers.
FIG. 5. The role of ΨΨΨC loop in complementation of psHIV-Phe. (A) Illustrations of the positions of the nucleotides mutated in mutants tRNA-U₅₆, tRNA-Δ₅₆, and tRNA-Δ₅₄Δ₅₆. (B) The psHIV-Phe rescue capacity of the indicated tRNA₆th mutants as measured by drug-resistant colony numbers.
FIG. 6. The secondary structures of the PBS and surrounding regions in wild type HIV-1 (HXB2 strain) (A) and psHIV-Phe (B) predicted by M-fold structure modeling program. The PBS regions are in bold. The loop regions in italic represent the PBS-downstream sequence complementary to the U4C loop sequence of tRNAs.
proposed interaction between TΨC loop and the PBS-downstream sequence (Fig. 5A). Strikingly, the resultant mutant tRNA-A_{56} almost completely lost the capacity to rescue psHIV-Phe. Furthermore, a C_{56} → U_{56} substitution at the same position resulted in a mutant (tRNA-U_{56}) with considerably greater virus-rescue capacity when compared to tRNA-A_{56} (Fig. 5B). Unlike A_{56}, U_{56} could base pair with G to restore the complementarity between TΨC loop and the PBS-downstream sequence. This result further supports the possibility of a functional interaction between TΨC loop of tRNA^{Phe} and the PBS downstream sequence of psHIV-Phe.

**DISCUSSION**

In this study, we have investigated the role of elements within the tRNA acceptor stem and TΨC stem-loop in the primer selection and use in HIV-1 in vivo, by testing the capacity of tRNA^{Phe} mutants to rescue a defective HIV-1 (psHIV-Phe) which relied on exogenous yeast tRNA^{Phe} for replication. The results demonstrate that, although the tRNA^{Phe} 3'-end unpaired sequence (ACCA) was not required for psHIV-Phe infectivity, elements within the tRNA^{Phe} acceptor stem structure were important for the rescue of psHIV-Phe. Furthermore, we have identified nucleotides within the TΨC stem-loop that were critical for rescue of the virus.

The results of our studies provide new information on the role that specific regions of the tRNA play in selection and use in HIV-1 replication. Our analysis focused initially on the acceptor stem of the tRNA molecule. Since tRNA^{Phe} without the 3' ACCA sequence could rescue psHIV-Phe, it would suggest that a single-stranded region at the tRNA 3' end is not required for the tRNA-PBS interaction. This conclusion is
based on the assumption that the deleted ACCA sequence was not added back by cellular enzymes following cotransfection. The 3'-end ACC sequence of mature eukaryotic tRNAs are not encoded by tRNA genes, instead, it is specifically added by the ATP(CTP):tRNA nucleotidyltransferases (CCA-adding enzymes) (24). The CCA-adding enzymes are distributed both in the nucleus and cytoplasm (24). Therefore, we could not rule out the possibility that the 3'-end CA sequence deleted in mutant tRNAΔCA might have been added back after its cotransfection. However, mutant tRNAΔACCA is an unlikely substrate for CCA-adding enzymes due to the deletion of 3' CCA along with the so-called discriminator nucleotide A73, an important identity determinant of the tRNA. To our knowledge, no CCA-addition to a tRNA without discriminator N73 has been reported. That the CCA-addition can take place with a tRNA^{Tyr} minihelix consisting of the acceptor stem and TψC stem-loop including the discriminator A73 but not with a version lacking the discriminator base suggests a substrate specificity of the CCA-adding enzymes (20). Although a specific discriminator adding activity has been identified in mitochondria, which repairs a mitochondrial tRNA lacking N73, due to the overlapping nature of mitochondrial tRNA genes, this discriminator repair activity is not found outside of the mitochondria (19). Therefore, the result from analysis of mutant tRNAΔACCA suggests that the 3' ACCA sequence of tRNA^{Phe} is not required for it to function as a reverse transcription primer. Thus, the remaining 14-nt complementarily between the tRNA and PBS was sufficient for selection and use in HIV infection. The 3'-end CCA sequence is universal among all mature eukaryotic tRNAs and is important for their natural function in protein synthesis. The lack of requirement for this sequence in the tRNA primer, coupled with our previous results that the D stem-loop of tRNA^{Phe}
was not required for complementation (26), may indicate that HIV-1 can use tRNAs that are not functional for protein synthesis. If this is the case, HIV-1 might have evolved to use such tRNAs so as to avoid competition with the cellular protein synthesis machinery. Further experiments will be needed to address this possibility.

Our studies also demonstrate that the acceptor stem of tRNA\textsuperscript{Phe} is important for rescue of psHIV-Phe. Both adding and removing nucleotides at the 5' end impaired the virus-rescue capacity of the tRNA. The actual role of the tRNA 5' end in the primer selection and use remains to be clarified. Our data have suggested that the base pairing in the acceptor stem involving the tRNA 5' end is important for this process. A cross-linking study showed that the 5' end of tRNA\textsuperscript{Lys,3} interacts with the C-terminal portion of HIV-1 RT (16). It is possible that this interaction is important for the tRNA primer use in HIV-1. However, it is unlikely that the requirement for the tRNA 5' end as a primer is sequence-specific, since the sequence of the 5' end is not conserved among tRNAs except the first G, and many different tRNAs can be used as reverse transcription primers for HIV-1 in vivo (7, 11, 14, 23). In addition, in the structure model of HIV-1 RNA-tRNA\textsuperscript{Lys,3} initiation complex, the 5' end of the tRNA was proposed to form a helix with the 5' strand of the TΨC stem (9, 10). Mutations of the tRNA 5' end then may also affect the formation of the functional viral RNA-tRNA complex. Further studies will be required to test this possibility.

A surprising result from our studies was the importance of the nucleotides in the TΨC stem-loop of tRNA\textsuperscript{Phe} (Fig. 5). This region of the tRNA includes a sequence complementary to the PBS 3' end and the PBS-downstream region (nt 201-205). We believe that our results have implications for understanding the selection process for the
tRNA primer. One possibility is that the tRNA TΨC loop interacts with the viral PBS 3' end and its downstream sequence, as an initial step in the tRNA-PBS binding. A model of HIV-1 RNA secondary structure suggested that the 3' end of the PBS and the PBS-downstream sequence were located in a loop region, whereas the 5' end of the PBS was located in a stem structure (Fig. 6) (9). If the model is correct, the proposed interaction between the PBS-downstream sequence and the tRNA TΨC loop might occur through a low-energy loop-loop interaction. This is attractive because of the lower energy required for initiation of the tRNA-PBS annealing, whereas an initiation from the PBS 5' end located in a stem would necessitate unwinding of the PBS prior to the initial interaction. Our results from tRNA Phe mutants, which would not favor the interaction of the TΨC loop with the PBS-downstream sequence, support the concept that this is an essential interaction. In vitro studies have demonstrated that HIV-1 NC protein promotes tRNA annealing to the viral RNA (1). Recent studies showed that NC protein does not promote the unwinding of tRNA Lys in the absence of viral RNA genome (4, 8). Additional experiments will be needed to determine whether the tRNA TΨC loop and viral PBS-downstream sequence complementarity facilitates an initial interaction that might trigger the NC protein to unwind the tRNA and promote PBS-annealing.

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DISCUSSION AND CONCLUSIONS

As the causative agent of the AIDS pandemic, HIV-1 has been a focus of intense investigation. Although a great deal of knowledge has been gained about HIV-1 replication in general, numerous aspects of the mechanism for HIV-1 reverse transcription still remain to be clarified. One of the critical, unsolved issues centers on how HIV-1 selects the tRNA primer for initiation of reverse transcription.

tRNA$^{\text{Lys}}$ is the natural primer for HIV-1 reverse transcription. The selective use of the tRNA primer is mainly determined by the PBS region of HIV-1. Substitution of the PBS to sequence complementary to alternative tRNAs resulted in viruses that used alternative tRNAs as primers (30, 65, 81, 128). However, the 3' terminal 18 nucleotides of a tRNA alone is not sufficient for the primer selection and use in HIV-1 in vivo; additional sequence and structural elements within the tRNA are required, as demonstrated by experiments in this dissertation. These investigations were carried out in an in vivo complementation system that allows the manipulation of the tRNA primer for a mutant HIV-1 (136). The results demonstrate that elements of the tRNA acceptor stem, TΨC stem-loop, and anticodon stem-loop, but not the D stem-loop, are important for the primer selection and use in HIV-1.
PBS is the Major Determinant in HIV-1 for the Selection and Use of the tRNA Primer (Paper #1)

In this research, a single-round infection system was used to study the replication efficiency and primer use for a mutant HIV-1 with an alternative PBS. The results revealed that HIV-1 with a PBS complementary to tRNA^{ile} (HIV-Ile) or tRNA^{His} (HIV-His) predominantly used the cognate tRNA to initiate reverse transcription in their first round of infection. These results further demonstrate that the complementarity between the tRNA primer and the PBS is the major determinant for the selection of the primer for HIV-1 reverse transcription. These HIV-1 mutants though are less efficient in replication compared to the wild-type virus that uses tRNA^{Lys,3} as the primer. Clearly, there are factors other than the tRNA-PBS interaction that are involved in the primer selection and use, which might include additional interactions between the tRNA primer and viral RNA and/or protein(s) and the availability of different tRNA species. A single use of tRNA^{Lys,3} as the primer was detected in the first-round infection of HIV-Ile. This result is correlated with a previous observation that HIV-1 with a PBS complementary to tRNA^{ile} reverted back to wild type more rapidly than the virus with a PBS complementary to tRNA^{His} (128). The 3'-end 18 nucleotides of tRNA^{Lys,3} differ from the same regions of tRNA^{ile} and tRNA^{His} in 9 and 7 nt positions, respectively. However, tRNA^{Lys,3} could form more base pairs with the PBS complementary to tRNA^{ile} than with the PBS complementary to tRNA^{His}, which might result in the lower capacity of HIV-Ile to exclude tRNA^{Lys,3} from initiating reverse transcription when compared to HIV-His. We speculate that two major factors are involved in the reversion of HIV-1 with an alternative PBS to wild type: the replication efficiency of the mutant virus compared to wild type and the ability of the virus to exclude tRNA^{Lys,3} from initiating reverse
transcription. If a mutant virus using an alternative tRNA primer can effectively exclude other tRNAs from initiating reverse transcription and replicates as efficiently as the wild-type virus, this virus probably will maintain the use of the alternative tRNA as primer. Even if this virus replicates slower than the wild type, since other tRNAs can not be used for reverse transcription, the virus probably will be able to maintain the alternative PBS for at least a long time. Conversely, if a virus using an alternative tRNA primer can not completely prevent tRNA$^{Lys,3}$ from binding to initiate reverse transcription and replicates slower than the wild-type virus, the revertant virus with a wild-type PBS will arise rapidly and eventually become the major species in the virus population. The combinations of these two factors probably dictate the reversion rate of the mutant viruses. Therefore, it is not surprising that HIV-Ile, which failed to prevent tRNA$^{Lys,3}$ from being a primer at the very first round of infection and also replicated slower than the wild-type virus, reverted to use tRNA$^{Lys,3}$ as the primer rapidly. HIV-1 with both A-loop and PBS complementary to tRNA$^{His}$ replicated more efficiently compared to the virus with only the PBS complementary to tRNA$^{His}$ in single round of infection. This improved replication efficiency along with the enhanced capacity to exclude tRNA$^{Lys,3}$, through specific A-loop interactions, contributes to the stable use of tRNA$^{His}$ as a primer by the virus with both the A-loop and PBS complementary to tRNA$^{His}$.

The major emphasis of this study was to determine whether a noninfectious HIV-1 with a PBS complementary to yeast tRNA$^{Phe}$ (psHIV-Phe) can be rescued by exogenous tRNA$^{Phe}$. Yeast tRNA$^{Phe}$ provided in trans did restore the infectivity of a psHIV-Phe. This result further confirms the PBS as the major determinant for tRNA primer selection and use in HIV-1. Additionally, the fact that HIV-1 not only can use
human tRNAs but also can use a yeast tRNA as primers in vivo indicates the absence of an element(s) unique in human tRNAs that might be required for HIV-1 primer selection and use. Both native tRNA\textsuperscript{Phe} (with modified bases) and in vitro synthesized tRNA\textsuperscript{Phe} (without modifications) can be used in transfection to rescue psHIV-Phe, suggesting that modified bases of the tRNA are probably not essential for the primer selection and use in HIV-1. Additional studies are needed to confirm this possibility by determining whether yeast tRNA\textsuperscript{Phe} is modified by mammalian enzymes after transfection. The unique aspect of the psHIV-Phe complementation system is that it allows manipulation of the tRNA primer for an HIV-1 in vivo, which was taken advantage of in this research to investigate elements of the tRNA that are important for HIV-1 primer selection and use.

The tRNA Anticodon and D Stem-Loops Play Different Roles in the Primer Selection and Use in HIV-1 (Paper #2)

Since HIV-1 could use many different tRNAs but not PBS-binding RNA oligomers as primers in vivo, we believe there are elements present in tRNAs but absent in PBS-complementary oligomers that are critical for the primer selection and use in HIV-1. In this research, we used yeast tRNA\textsuperscript{Phe} as a model to identify these important elements. The results indicate that the anticodon and TYC stem-loops of tRNA\textsuperscript{Phe} are important for the primer selection and use, whereas the D stem-loop is not required for this process.

The anticodon stem-loop region is important for psHIV-Phe replication, since deletion of the anticodon stem and/or loop sequence severely impaired the virus-rescue capacity of the tRNA. However, mutations introduced to either disrupt or restore the stem-loop structure of the anticodon region did not affect the tRNA from rescuing the
virus, suggesting the native secondary structure of the anticodon stem-loop is not required for the primer selection and use in HIV-1. Studies on the retrotransposon Ty1 have also led to a similar conclusion, since mutations that disrupt the anticodon stem structure of the tRNA primer for Ty1 did not affect Ty1 replication (69). It would be interesting to find out whether the lack of requirement for the native anticodon stem-loop structure in the tRNA primer is also the case for other retroviruses and retrotransposons.

A possible explanation for the importance of the anticodon stem-loop in psHIV-Phe replication would be in its sequence. Analysis of the anticodon stem-loop sequence of different tRNAs revealed only a single conserved nucleotide, $U_{34}$. Among the tRNAs that can be used to initiate productive HIV-1 reverse transcription in vivo, tRNA$^{\text{Lys,3}}$, tRNA$^{\text{Met}}$, tRNA$^{\text{Trp}}$, tRNA$^{\text{Lys,2}}$, and yeast tRNA$^{\text{Phe}}$ have a very similar sequence in the anticodon stem-loop (except the anticodon); whereas the anticodon stem-loop sequence of tRNA$^{\text{His}}$, tRNA$^{\text{Ile}}$, and tRNA$^{\text{Pro}}$ are very different from tRNA$^{\text{Lys,3}}$. Since all these tRNAs can be used as primers for HIV-1, the basic requirement for the anticodon stem-loop in primer selection and use might not be sequence-specific. Further investigation is clearly needed to define the role of the anticodon stem-loop in the primer selection process. A possibility might be that the anticodon stem-loop contributes to the overall structure formation or stability of the tRNA. It is important to note that, although the specific sequence within the anticodon stem-loop is probably not required, it might still influence the efficiency of reverse transcription initiation and strand transfer through interacting with the viral U5 and U3 sequences (16).

Strikingly, deletion of the D stem-loop did not affect the tRNA from rescuing psHIV-Phe. As discussed in the paper, this result suggests that the native tertiary
structure of the tRNA is not critical for the primer selection and use in HIV-1, since the D loop is essential for the tertiary folding of the tRNA. The role of D stem-loop in protein synthesis remains unclear. Certain mitochondrial tRNAs do not contain the D stem-loop, which might indicate that a tRNA structure functional in protein synthesis could be formed without the D stem-loop under certain circumstances. Interestingly, our data demonstrate that tRNA\textsuperscript{Phe} without the D stem-loop also meets the sequence and structure requirements for the tRNA primer selection and use in HIV-1. Further studies will be needed to determine whether this result can be generalized into other retroviruses and retrovirus-like elements. The D stem-loop is located near the "elbow" of the L-shaped tRNA molecule, which may make it less accessible and therefore less involved in interactions with other molecules that bind with the tRNA. The structure of tRNA without D stem-loop remains unknown. Solving this structure will help to understand the structure requirements for the tRNA in HIV-1 primer selection.

The D stem-loop and anticodon stem-loop are also called the bottom half of the tRNA, which are proposed as later additions to the tRNA structure during the evolution. The top half of the tRNA, consisting of the acceptor stem and T\PsiC stem-loop, probably resembles the ancient tRNA-like structures. Maizels and Weiner have proposed that tRNA-like structures arose as genomic tags at the 3' termini of RNAs and served as a primer and recognition element for RNA replication in the RNA world (89). The use of tRNAs as primers by contemporary retroviruses may reflect the ancient function of tRNA-like structures in replication. The lack of requirement for the D stem-loop and the native anticodon stem structure in psHIV-Phe replication might imply that the tRNA bottom half is less involved in the HIV-1 replication. Indeed, our studies on of tRNA\textsuperscript{Phe}
suggest that the top half is relatively more important for the replication of psHIV-Phe, as discussed in the following section.

The Top Half of the tRNA is Important for Primer Selection and Use in HIV-1 (Paper #3)

This paper focuses on the role of the top half of tRNA\(^{\text{Phe}}\) in primer selection and use in HIV-1. The top half of the tRNA consists of the acceptor stem and T\(\Psi\)C stem-loop. Our data indicate that the 5’ end of the tRNA located in the acceptor stem is critical for the infectivity of psHIV-Phe virus. Both adding and removing nucleotides at the tRNA 5’ end impaired the virus-rescue capacity. These results are unlikely due to a sequence-specific requirement for the tRNA 5’ end, since the 5’-end sequence is not conserved among tRNAs. A more plausible explanation would be that mutations at the tRNA 5’ end affect the structure of the acceptor stem. This possibility is supported by the results from tRNA\(^{\text{Phe}}\) mutants with point mutations to influence the acceptor stem structure (tRNA-U_2A_4 and tRNA-A_2C_4).

The 3’ end of the tRNA is also located at the acceptor stem. Our data suggest that the 3’ end of the tRNA primer for HIV-1 does not have to be CCA, a 3’-end sequence universal among tRNAs. The capability of HIV-1 to use immature or impaired tRNA molecules as primers may benefit the virus by avoiding competition with the translation machinery for mature and functional tRNAs. In another experiment, the virus could use a tRNA primer with four but not nine additional nucleotides at the 3’ end. In contrast, tRNA mutants with more than nine extra nucleotides could be used to initiate reverse transcription by HIV-1 RT in vitro (102). The M-fold program predicted that the tRNA mutants with additional nucleotides form the same cloverleaf structure except in the
length of the extended 3' end. These data together suggest that an over extended tRNA 3' end might not prevent the tRNA from initiating reverse transcription, but it could prevent the tRNA from being selected as a reverse transcription primer for HIV-1.

The TΨC stem-loop of tRNA\textsuperscript{Phe} is crucial for the infectivity of psHIV-Phe virus. The 3’ portion of the TΨC stem-loop is part of the tRNA sequence that interacts with the PBS. Its immediate-upstream sequence is the TΨC loop sequence (5'-'3'TΨCRA\textsubscript{A58}-3'), R as A or G) that is highly conserved among tRNAs. Our results indicate that the TΨC sequence is critical for the primer selection and use in psHIV-Phe. An HIV-1 U5 sequence (5'-UUUUAGUC-3') was previously proposed to interact with the TΨC stem-loop of tRNA\textsuperscript{Lys,3} (5'-GGTΨCA\textsubscript{A58}-3') (proposed base-pairing nucleotides are italicized) (91). We have identified a sequence downstream of the PBS that shares complementarity with the TΨC loop. Our data support a potential interaction of the TΨC loop with the PBS-downstream sequence but not with the U5 sequence. A tRNA\textsuperscript{Phe} mutant (tRNA-A\textsubscript{56}) with a mutation that disrupts base pairing between the TΨC loop and the PBS-downstream sequence but enhances the complementarity between the TΨC loop and the U5 sequence failed to rescue the virus. Furthermore, a mutation at the same position (C\textsubscript{56}→U\textsubscript{56}) to restore the complementarity between TΨC loop and the PBS-downstream sequence without affecting the complementarity between TΨC loop and the U5 sequence resulted in improved virus-rescue capacity when compared to tRNA-A\textsubscript{56} (Fig. 5A and B). These results disagree with the speculative interaction between the TΨC loop and the U5 sequence (91) and yet support the possibility of a functional interaction between the TΨC loop of tRNA\textsuperscript{Phe} and the PBS downstream sequence of psHIV-Phe.
The TΨC loop sequence (5'-54ΨCRA58-3', R as A or G) is highly conserved among tRNAs including tRNA$^{\text{Lys3}}$, the natural primer for HIV-1 reverse transcription. A 5-nucleotide sequence complementary to the tRNA$^{\text{Lys3}}$ TΨC-loop (5'-UUGA'A-3', occasionally as G) was identified downstream of the PBS in most HIV-1 isolates (including the HXB2-gpt clone). Previous studies indicated that the HIV PBS-downstream sequence is important for reverse transcription initiation (73, 83). Based on this information and our data, we hypothesized that the TΨC loop of the tRNA primer for HIV-1 reverse transcription interacts with the PBS-downstream sequence of the viral RNA genome. This proposed interaction may facilitate the anchoring or positioning of the tRNA primer onto the viral RNA genome. It might help to bring together the 3' end of PBS and its complement in the tRNA primer, which might subsequently lead to the tRNA-PBS annealing, starting with the initial binding of the PBS 3' end to the 5' end of the PBS-binding sequence of tRNA (in the TΨC loop), followed with invasion of the TΨC stem and the acceptor stem by the PBS. Further investigations are needed to verify the proposed interaction.

The UUGAA sequence downstream of the PBS is also conserved in HIV-2 and simian immunodeficiency virus (SIV) RNA genomes. Interestingly, we noticed that the sequence immediately downstream of the PBS in several other lentiviruses as well as endogenous murine leukemia viruses also share complementarity with the tRNA TΨC loop. These viruses include equine infectious anemia virus (5'-CUGAG-3'), feline immunodeficiency virus (5'-UUGAU-3'), and MLV-like endogenous virus (5'-UUGGA-3'). The implication of these coincidences remains to be determined.
We can not rule out the possibility that the importance of the TΨC loop might also be due to its interaction with viral protein(s). The unique and yet highly conserved TΨC loop of tRNAs may serve as a signature element for the general selection of tRNAs from cellular RNA pool by HIV-1. In vitro, both tRNAs and oligonucleotides complementary to the PBS can be used to initiate reverse transcription. Whereas, in vivo, HIV-1 can use different tRNAs but not the oligonucleotides as reverse transcription primers, indicating the presence of elements conserved in different tRNAs that are required for the primer selection in vivo. Structural and sequence features that are common among tRNAs include the L-shape tertiary structure, the 3'–end CCA sequence, and sequences of the D loop and TΨC loop. Our data suggest that the TΨC loop is the critical element among these conserved features for the primer selection and use in HIV-1. Therefore, it is possible that the TΨC loop could serve as a signature sequence for viral factors, such as the RT portion of Gag-Pol to recognize. This possibility is supported by in vitro studies indicating direct interaction between RT and the tRNA TΨC loop (102, 132). Further studies are needed to address this possibility.

Final Remarks

The selection and use of the tRNA primer in HIV-1 is a complex process that remains largely unknown. Previous studies have indicated that the selection of the tRNA primer molecule is probably a separate process from the selective enrichment of tRNAs into virions (138). How is the actual tRNA primer selected in HIV-1? The viral RNA genome, especially the PBS region, is likely to be involved in this process, since HIV-1 can simultaneously switch to use alternative tRNAs as primers when only the PBS region
is mutated. Additionally, mutant HIV-1 which stably used tRNA$^{\text{His}}$ as the primer maintained a wild-type RT and NC proteins, indicating that RT and NC are not in charge of the actual tRNA primer selection (138). However, the complementarity between a primer and the PBS is not sufficient for the primer selection and use in HIV-1. Additional interactions between the tRNA primer and the viral RNA genome are probably required to facilitate this process. Indeed, our studies demonstrate that tRNA elements outside of the PBS-binding sequence of the tRNA are required for the primer selection. We have shown that the tRNA acceptor stem structure, TYC loop sequence, and element(s) in the anticodon stem-loop are among the essential tRNA elements for the primer selection and use in HIV-1. This information will help to identify interactions between the tRNA primer and the viral factor(s) that are important for the primer selection in HIV-1.

Where the HIV-1 primer selection takes place remains one of the major unanswered questions. The PBS-tRNA interaction would be required to ensure the specificity of primer selection. For this interaction to occur, partial unwinding of the secondary structures of the viral RNA and tRNA molecules is likely needed, which presumably involves the NC portion of Gag/Gag-Pol. It is not known whether the tRNA-PBS interaction takes place in the nucleus or the cytoplasm or within the virion. Determination of the cellular distribution of Gag/Gag-Pol would aid in understanding the location of primer selection. Recently, both nuclear import and export signals have been identified in MA that involves in directing HIV-1 genomic RNA to the virus assembly site (33). It is tempting to speculate that these signals might enable Gag/Gag-Pol to shuttle between the cytoplasm and nucleus to direct the unspliced viral RNA toward the
virus assembly site. This speculation currently does not have any supporting evidence and needs to be tested. If this is the case, it may be possible that Gag/Gag-Pol in the nucleus might also promote the interaction of the tRNA primer with the PBS of the viral RNA destined for virion assembly. Alternatively, the primer selection may occur in the cytoplasm. The viral genomic RNA might interact with the tRNA primer in cytoplasm, possibly with the help from Gag/Gag-Pol, and be directed toward the virus assembly pathway. A second but less likely alternative would be that viral protein(s) nonspecifically packages tRNAs into the virion; subsequently the selection of the actual tRNA primer occurs within the virion through tRNA-PBS interaction.

The psHIV-Phe complementation system we established is a useful tool that can be used to study the location and other aspects of the HIV-1 primer selection and use in the future. For instance, analyzing the psHIV-Phe rescue capacity of tRNA\textsuperscript{Phe} mutant(s) with a defect in nuclear export will provide useful information in understanding the location of primer selection in HIV-1; using labeled tRNA\textsuperscript{Phe} in the system will also aid in study of the tRNA and viral RNA interaction. Additionally, since our results show that tRNA\textsuperscript{Phe} directly transfected into cells can be used by the progeny virus as the primer, it would be useful to determine whether the transfected tRNA stays in the cytoplasm. If this is the case, then the tRNA primer selection for HIV-1 could occur in the cytoplasm. Further investigation is needed to address this possibility.

The initiation of HIV-1 reverse transcription is a target for drug design. Molecules mimicking the tRNA were designed to inhibit HIV replication by either re-directing the initiation site of reverse transcription (85) or by out-competing the tRNA primer for the viral PBS or RT (61, 79). Elucidation of elements within the tRNA primer
critical for primer selection and use in HIV-1 will aid in designing these new inhibitors of HIV-1 replication.


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83. **Liang, C., L. Rong, R. S. Russell, and M. A. Wainberg.** 2000. Deletion mutagenesis downstream of the 5' long terminal repeat of human immunodeficiency virus type 1 is compensated for by point mutations in both the U5 region and gag gene. J. Virol. 74:6251-6261.


Name of Candidate  Qin Yu

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name  Signature
Casey D. Morrow, Chair
Eric Hunter
Andrew Ball
Stephen L. Hajduk
Beatrice H. Hahn

Director of Graduate Program

Dean, UAB Graduate School

Date 1/16/02